DOCTOR OF PHILOSOPHY

Development and validation of a cell based model of insulin resistance and investigation into the intracellular molecular defects induced by diabetes and obesity

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University of Dundee

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By

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Declarations

I hereby declare that the following thesis is based on the results of my own investigations conducted by myself and that this thesis is of my own composition. Work other than my own is clearly indicated in the text by reference to the researchers or their publications. This dissertation has not in whole, or in part, been previously presented for a higher degree.

Christopher J Schofield

I certify that Christopher Schofield has spent the equivalent of nine terms in research work in the Biomedical Research Institute, School of Medicine, University of Dundee and that he has fulfilled the conditions of the Ordinance General No. 14 of the University of Dundee and is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy.

Dr. Calum Sutherland

Summary

A reduction in the sensitivity of tissue to insulin is termed insulin resistance. In the clinic this condition is associated with obesity and inactivity and often leads to the development of type 2 diabetes. A major focus of antidiabetic therapy is to develop novel interventions to alleviate insulin resistance. However, the initial physiological and molecular defects in the development of insulin resistance remain elusive. This knowledge would greatly aid the development of novel and more effective insulin sensitisers.

In an effort to improve the understanding of insulin resistance this thesis establishes that culturing liver cells in sera from obese diabetic patients reduces the ability of insulin to repress the key gluconeogenic gene, phosphoenolpyruvatecarboxykinase (PEPCK). Cells cultured in serum from obese diabetic human subjects exhibited defective PEPCK mRNA suppression by 0.1 and 0.5 nM insulin compared to cells cultured in control serum ($p<0.0001$), representing a shift to the right of the insulin dose response curve. Classification of human sera, using the response of the cell model following incubation with the sera, was actually more reliable than any single clinical biomarker at establishing whether the serum came from a volunteer with insulin resistance. This suggests that the cell model could be developed as a means to classify insulin resistance in the human population more reliably than simply measuring fasting glucose.

The system was developed and optimised as a cell based humanised model of insulin resistance to aid the search for a biomarker for the development of obesity related insulin resistance. However, there was no linear relationship between any single biomarker and the resistance causing ability of the sera. Interestingly, cells cultured

chronically in the presence of fetal calf serum supplemented with 5 pM insulin (the average increase in insulin between cases and controls) also exhibited reduced suppression of PEPCK by 0.1 and 0.5 nM insulin compared to controls ($p=0.03$ and 0.01 respectively). This has major implications for the understanding of how insulin resistance may develop. It suggests that minor increases in insulin release from beta cells, or minor loss of insulin clearance in the liver that elevate plasma insulin are potential initiating mechanisms for insulin resistance (at least in liver). Of course there may be many ways to initiate insulin resistance *in vivo*, but establishing the relative importance of the beta cell and the liver as an initial site for the development of insulin resistance is clearly important for effective intervention. Subsequent to the generation of insulin resistance in culture I could not detect significant differences in the response of the major post-receptor insulin signalling pathway components, between cells cultured under standard conditions and those cultured chronically in 5 pM insulin. Therefore the mechanism underlying this reduced insulin action on PEPCK gene transcription remains unclear.

I then went on to develop reporter cell lines both for use in the study of the regulation of hepatic gene transcription by insulin and also as a potential screen for effective insulin sensitisers. Unfortunately the reporter cell lines did not turn out to be useful as hoped, as the reporter genes did not develop insulin resistance in response to chronic exposure to 5 pM insulin. In addition there were some differences between the reporter genes and endogenous genes in response to specific signalling inhibitors. This questions their suitability for the purposes proposed.

Finally, I examined the signalling connections between the class of insulin sensitiser known as biguanides, and DNA repair mechanisms, as an initial characterisation of

molecular links between diabetes and cancer. I established that inhibiting the DNA repair enzyme ATM reduces the phosphorylation of the biguanide target, AMPK in response to these drugs. However, although inhibition of ATM reduced biguanide suppression of G6Pase it had little effect on the regulation of PEPCK gene transcription by the drugs. This is consistent with AMPK not being the key mediator of biguanide regulation of PEPCK gene transcription and suggests that biguanide regulation of G6Pase and PEPCK gene transcription is mediated through distinct signalling pathways.

In summary, I have developed a cell based model of insulin resistance that relies on factor (s) present in serum from humans with diabesity, and thus should be useful as a screen for more effective insulin sensitisers targeted at the population that donates the serum. It is likely that one of the factors responsible for generation of resistance is insulin itself as chronic exposure to low levels (albeit higher than background), of insulin reduces insulin sensitivity of the cells. The molecular details of the development of insulin resistance remain elusive as none of the major signalling pathways appear to be defective in the cells that have developed reduced insulin regulation of PEPCK. However, the data raise the intriguing possibility that chronic but mild hyperinsulinemia due to defective insulin secretion or clearance is an initial step in the development of insulin resistance. Hence, reducing insulin secretion (as opposed to current strategies of inducing insulin secretion) may be a more effective therapy for prevention of the development of insulin resistance. Finally, elements of the DNA repair pathways such as ATM may impinge on pathways that affect insulin sensitivity, including the biguanide target AMPK.

Publications arising from work in this thesis

Common variants near ATM are associated with glycemic response to metformin in type 2 diabetes.

GoDARTS and UKPDS Diabetes Pharmacogenetics Study Group; Wellcome Trust Case Control Consortium 2, Zhou K, Bellenguez C, Spencer CC, Bennett AJ, Coleman RL, Tavendale R, Hawley SA, Donnelly LA, **Schofield C**, Groves CJ, Burch L, Carr F, Strange A, Freeman C, Blackwell JM, Bramon E, Brown MA, Casas JP, Corvin A, Craddock N, Deloukas P, Dronov S, Duncanson A, Edkins S, Gray E, Hunt S, Jankowski J, Langford C, Markus HS, Mathew CG, Plomin R, Rautanen A, Sawcer SJ, Samani NJ, Trembath R, Viswanathan AC, Wood NW; MAGIC investigators, Harries LW, Hattersley AT, Doney AS, Colhoun H, Morris AD, Sutherland C, Hardie DG, Peltonen L, McCarthy MI, Holman RR, Palmer CN, Donnelly P, Pearson ER.

Nat Genet. 2011 Feb;43 (2):117-20.

Generation, validation and humanisation of a novel insulin resistant cell model.

Logie L, Ruiz-Alcaraz AJ, **Schofield CJ**, Hundal HS, Feuerstein GZ, Brady JD, Crowther D, Tommasi AM, Grierson CE, Shepherd B, Morris AD, Hansen MK, Pearson E, Sutherland C.

Biochem Pharmacol. 2010 Oct 1;80 (7):1042-9.

Abbreviations

aa	Amino acid
ABD	Adaptor binding domain
ACC	Acetyl-CoA carboxylase
AF	Accessory factor
AICAR	5-Aminoimidazole-4-carboxamide riboside
AMP	Adenosine monophosphate
AMPK	AMP activated protein kinase
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
BMI	Body mass index
bpm	Beats per minute
BRCA1	Breast cancer 1
cAMP	3'-5'-cyclic adenosine monophosphate
C/EBP	CCAAT-enhancer-binding protein
CAMKK/CKK1	Calcium/calmodulin stimulated kinase kinase
CBS	Cystathionine-β-synthase
CDKAL1	CDK5 regulatory subunit associated protein 1-like 1
cDNA	Complementary DNA
Cm	Centimetres
COUP-TF	Chicken ovalbumin upstream promoter transcription factor
CRE	cAMP response element
CREB	cAMP response element-binding
CRP	C-reactive protein
CRU	cAMP response unit
C-terminal	Carboxy-terminal
dAF	Distal accessory factor
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
DNA-PK	DNA dependent protein kinase
EC50	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ET-1	Endothelin-1
FANC-D2	Fanconi anemia group D2 protein
FAS	Fatty acid synthase
FATP1	Fatty acid transport protein 1
FFA	Free fatty acid
FGF	Fibroblast growth factor
FOXA	Forkhead Box A
FOXO	Forkhead box O
Fru 1,6, Pase	Fructose-1,6,-bisphosphatase
FTO	Fat mass and obesity-associated protein
G6P	Glucose-6-phosphate
G6Pase	Glucose-6-phosphatase
GDP	Guanosine diphosphate
GK	Glucokinase
GLUT	Glucose transporter

Grb2	Growth factor receptor-bound protein 2
GRE	Glucocorticoid response element
GRK2	G protein-coupled receptor kinase
GRU	Glucocorticoid response unit
GS	Glycogen synthase
GSK-3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
HbA1c	Glycated Haemoglobin
HDL	High density lipoprotein
HFD	High fat diet
HHEX	Hematopoietically-expressed homeobox protein
HMGCo-A	3-hydroxy-3-methylglutaryl-coenzyme A
HNF	Hepatocyte nuclear factor
HOMA-IR	Homeostatic model assessment of insulin resistance
IGF-1	Insulin-like growth factor-1
IGF2BP2	Insulin-like growth factor 2 mRNA-binding protein 2
IGFBP-1	Insulin-like growth factor binding protein-1
IKKB	Inhibitor of nuclear factor kappa-B kinase subunit beta
IL	Interleukin
IP	Immunoprecipitation
IP3	Inositol trisphosphate
IR	Insulin receptor
IRS	Insulin receptor substrate
JAZF1	Juxtaposed with another zinc finger protein 1
JNK	c-Jun N-terminal kinase
KCNJ11	Kir6.2 encoding gene
Kg	Kilograms
LDL	Low density lipoprotein
LKB1	Ser/Thr kinase 11
LPL	Lipoprotein lipase
MAPK	Mitogen activated protein kinase
MAPKK/ MEK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
MCP	Monocyte chemotactic protein
Minutes	Minutes
mmHg	Millimetres of mercury
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NBS1	Nibrin
NF-1	Nuclear factor 1
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NOTCH2	Neurogenic locus notch homolog protein 2
N-Terminal	Amino-terminal
PCOS	Polycystic ovarian syndrome
PCR	Polymerase Chain Reaction
PDK1	3-phosphoinositide dependent protein kinase-1
PEPCK	Phosphoenolpyruvate Carboxykinase
PFK	Phosphofructokinase
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-α

PH	Pleckstrin homology
PI 3-K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	Protein Kinase A
PKB	Protein Kinase B
PKC	Protein Kinase C
PP2A/C	Protein phosphatase 2A/C
PPARγ	Peroxisome proliferator-activated receptor gamma
PTB	Protein Tyr binding
PtdIns	Phosphatidylinositol
PTEN	Phosphatase and tensin homolog
PTP1B	Protein-Tyr phosphatase 1B
RAR	Retinoic acid receptor
RBD	Ras binding domain
RNA	Ribonucleic acid
RSK	Ribosomal S6 kinase
RXR	Retinoid X receptor
S6K	S6 kinase
SGK	Ser/Thr-protein kinase SGK
SGLT	Sodium dependent glucose transporter
SH2	Src homology 2
SHP	Small heterodimer partner
SHP2	SH2 domain-containing Tyr phosphatase-2
SIK	Salt inducible kinase
SIRT1	Sirtuin 1
SLC30A8	Solute carrier family 30 (zinc transporter), member 8
SMC1	Structural maintainance of chromosomes 1
SREBP-1c	Sterol regulatory element-binding protein 1c
TCF7L2	Transcription factor 7-like 2
TIRE	Thymine-rich insulin response element
TNF	Tumour necrosis factor
TORC2	Transducer of regulated CREB activity 2
TR	Thyroid hormone receptor
v/v	Volume/volume
w/v	Weight/volume
WFS1	Wolframin

Amino Acid Code

Amino Acid	**Three Letter Code**	**One Letter Symbol**
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
IsoLeucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	val	V

Chapter 1. Introduction

1.1. Insulin

Insulin is a 51 amino acid (aa) polypeptide hormone produced in the β-cells of the pancreatic islets of Langerhans where it is transcribed from the INS gene (Joshi et al., 2007). There is significant conservation across species. Human insulin consists of a 21 aa A chain and a 30 aa B chain linked by a pair of disulphide bonds (Joshi et al., 2007) In solution, insulin forms dimeric structures due to hydrogen bonds between the C termini of the B-Chains. (Joshi et al., 2007) The hisitidine residues of the insulin molecules readily bind to zinc ions to form highly stable hexamers.

Insulin synthesis initiates with translation of a single polypeptide chain of preproinsulin. During processing in the endoplasmic reticulum, the signal peptide is cleaved leaving proinsulin (Joshi et al., 2007). Proinsulin consists of the aforementioned N-terminal B chain and C-terminal A chain linked by C peptide. The C-peptide is removed by two endopeptidases, namely prohormone convertase 1 and 2 followed by carboxypeptidase E to leave the separate A and B chains linked only by disulphide bridges as described above (Joshi et al., 2007).

1.1.1. Insulin Secretion

Glucose enters β-cells of the pancreatic islets of Langerhans through the glucose transporter GLUT2 (Joshi et al., 2007). The Km value of GLUT2 for glucose lies within the physiological range of transition between fasted and fed plasma glucose levels allowing it to act as a glucose sensor. Once glucose enters the cell it is metabolised to glucose-6-phosphate by hexokinase and enters the glycolytic pathway (Joshi et al., 2007). The formation of ATP closes ATP sensitive potassium channels, Kir 6.2, leading to depolarisation of the cell and the opening of voltage gated calcium channels. Calcium

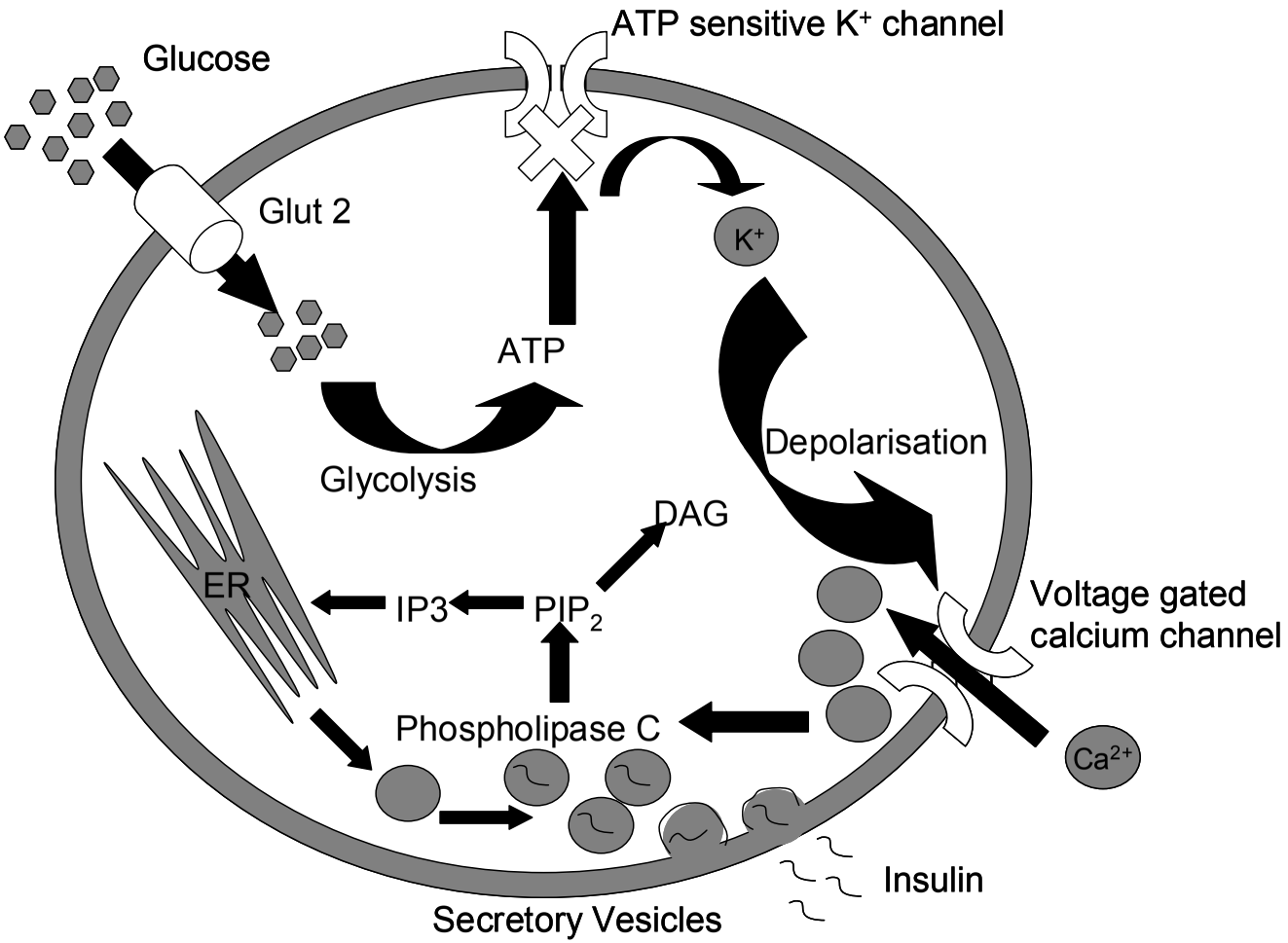


Figure 1.1 Mechanism of insulin secretion in β-Cell. **Glut2** = glucose transporter 2, **ATP** = adenosine triphosphate, $\mathbf{K^+}$ = potassium ions, $\mathbf{Ca^{2+}}$ = calcium ions, **DAG** = diacylglycerol, $\mathbf{PIP_2}$ = phosphotidylinositol bisphosphate, $\mathbf{IP_3}$ = inositol trisphosphate, **ER** = endoplasmic reticulum

activates phospholipase C, which in turn, converts phosphtidylinositol (4,5) bisphosphate (PIP2) into inositol trisphosphate (IP3). This leads to the release of insulin containing secretory vesicles from the endoplasmic reticulum which then fuse with the cell membrane allowing the contents to enter the circulation (Figure 1.1) (Joshi et al., 2007).

1.2. Clinical Insulin Resistance

Insulin resistance can be defined as a diminished response of a target tissue to any given quantity of insulin, and in clinical practice, this would lead to a lesser reduction in circulating glucose level per unit of insulin. This was first shown in 1936 when those who developed diabetes later in life tended to be resistant to insulin (Himsworth, 1936). This concept subsequently became central to the development of a number of disease states. A number of clinical measures of insulin resistance have been developed. As the development of insulin resistance is so closely related to obesity, it is of no surprise that body mass index (BMI) and waist circumference correlate with the level of insulin resistance (Farin et al., 2005). Furthermore, raised fasting insulin levels are also present in insulin resistance. However, the presence of these 2 factors shows the presence of insulin resistance, but does not allow for the quantification of said resistance.

The gold standard measure of insulin sensitivity is the hyperinsulinaemic euglycaemic clamp (DeFronzo et al., 1979). This is performed by intravenous infusion of a constant rate of insulin alongside a variable rate of glucose to maintain the blood sugar between 5 and 5.5 mmol/l. The rate of glucose required can be used to measure insulin sensitivity (DeFronzo et al., 1979). Those requiring high rates of glucose to maintain blood sugar within the normal range are insulin sensitive and those requiring low levels are insulin resistant and this can be quantified by the glucose infusion rate or total

glucose disposal (M-value). This test is rarely performed in clinical practice due to the time taken and resources requires.

A simpler test is an insulin tolerance test. After an overnight fast, the subject is exposed to insulin (0.1 units/kg) followed by repeated venous sampling for glucose. The slope of plasma glucose values correlates highly with the M-value of a hyperinsulinaemic-euglycaemic clamp (Bonora et al., 1989). However, in clinical practice the length of time taken for these tests renders them impractical.

Therefore, due to the impracticality of formal testing, a number of mathematical models of insulin sensitivity have been developed. The best known of these is the homeostatic model assessment of insulin resistance (HOMA-IR) (Matthews et al., 1985). By comparing the levels of fasting plasma glucose and insulin, one can quantify the level of insulin resistance in an individual and correlates well with the values seen in euglycaemic clamp studies (Matthews et al., 1985). HOMA-IR is calculated thus

$$\text{HOMA-IR} = \frac{\text{Glucose (mmol/l) x Insulin mU/l}}{22.5}$$

1.3. Diseases of Insulin Dysfunction.

1.3.1. Type 2 Diabetes

Type 2 Diabetes is a condition of glucose intolerance arising from a combination of insulin resistance and relative insulin deficiency resulting in enhanced hepatic glucose output. Although type 2 diabetes is often referred to as a single entity it is a heterogeneous group of conditions accounting for 90-95% of all diabetes, with some patients having a predominantly insulin resistant profile, and others insulin deficiency. However, it is widely accepted that the development of insulin resistance precedes

insulin deficiency. In contrast, patients with type 1 diabetes have an absolute deficiency of insulin.

As patients become insulin resistant, there is a consequent rise in insulin production by pancreatic β-cells to overcome the resistance. To enhance insulin output, β-cells become hypertrophic followed by a gradual decrease in secretory ability leading to the development of relative insulin deficiency and type 2 diabetes. The decrease in secretory capacity manifests as post-prandial hyperglycaemia in the first instance, but progressive β-cell failure leads to worsening hyperglycaemia. It is at this point, when approximately 60% of a patients β-cell mass has been lost that type 2 diabetes manifests clinically (Butler et al., 2003). Hepatic insulin resistance, in combination with insulin deficiency also leads to increased hepatic gluconeogenesis which further exacerbates hyperglycaemia so that it occurs in the fasting state (Figure 1.2). The reduced effect of insulin on its target tissues also results in an adverse metabolic profile, with high levels of LDL-cholesterol and triglycerides along with low levels of HDL-cholesterol. When combined with hypertension, hyperglycaemia and microvascular disease, this leads to a vast increase in the levels of macrovascular disease within the diabetic population. This atheromatous vascular disease is the principal cause of morbidity and mortality and is currently considered a coronary vascular disease equivalent in the middle aged and older diabetic patient (Grundy et al., 2004). Hyperglycaemia also causes the microvascular complications associated with diabetes, namely retinopathy, neuropathy and nephropathy (UK Prospective Diabetes Study (UKPDS) Group, 1998). There are also links between type 2 diabetes and the development of cancers and Alzheimer's dementia (Vigneri et al., 2009, Biessels et al., 2006).

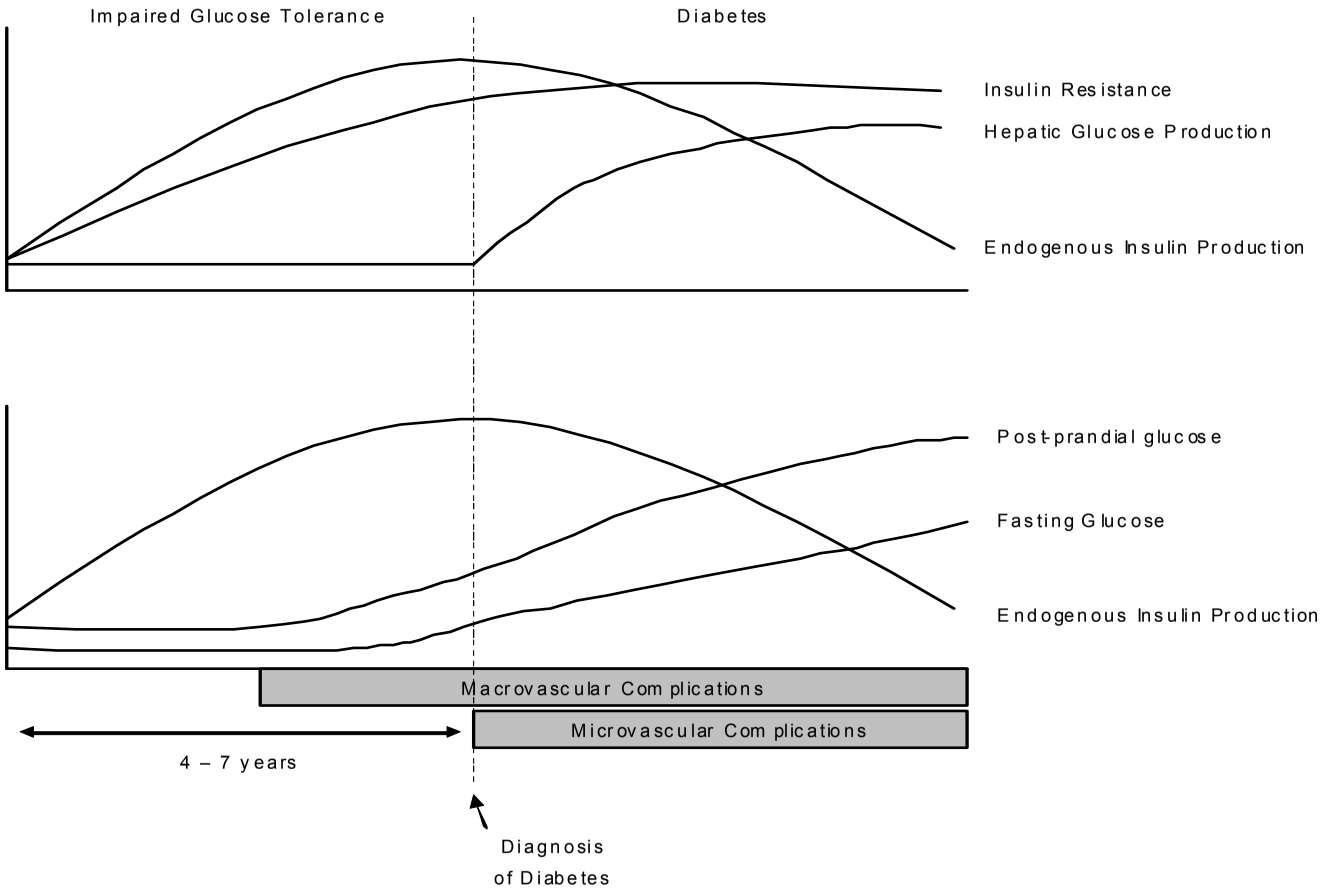


Figure1.2 The pathophysiology of insulin resistance, diabetes and complications. (Ramlo-Halsted and Edelman, 2000)

1.3.2. Non-Alcoholic Fatty Liver Disease

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in the western world affecting around 20% of the population (McCullough, 2006, Bedogni et al., 2007) . It covers a range of hepatic pathological changes from simple accumulation of triglycerides in hepatocytes (steatosis) through to fat accumulation with inflammation with or without fibrosis or cirrhosis (steatohepatitis) without alcohol as a causative factor. The progression from simple steatosis to non alcoholic steatohepatitis (NASH) shows an increasing severity of disease and insulin resistance is either a cause or consequence of the fat accumulation in the liver (Seppala-Lindroos et al., 2002, Vanni et al., 2010). The prevalence of NASH is only 2-3% in non obese individuals (Neuschwander-Tetri and Bacon, 1996) but this rises to up to 50% in the morbidly obese (Silverman et al., 1990). Approximately 10% of patients with NAFLD will progress to NASH over the next 10 years, and between 5 and 25% of these will develop cirrhosis and up to 50% will die from liver disease (McCullough, 2005, Matteoni et al., 1999). Furthermore, around 10% of those with cirrhosis will go on to develop hepatocellular carcinoma (McCullough, 2005).

1.3.3. Polycystic Ovarian Syndrome (PCOS)

The cluster of obesity, poor fertility, hirsutism and acne is known as PCOS and affects between approximately 4 and 18% of women of reproductive age (Knochenhauer et al., 1998, March et al., 2010). The majority of those with PCOS are also insulin resistant (Dunaif et al., 1989). Therefore, the risk of developing diabetes in these individuals is much greater with a conversion rate of between 1 and 5% per year (Legro, 2006). Multiple defects in the insulin signalling pathway have been demonstrated in those with PCOS, with changes in glucose uptake, attenuation of extracellular signal related kinase (ERK)1/2 (Chapter 1.6.7) phosphorylation in skeletal muscle, decreased insulin receptor

substrate (IRS)-1 (1.6.2) expression and phosphorylation and decreases in insulin stimulated Phosphoinositide 3-Kinase (PI 3-K) (1.6.3) activity to name a few (Diamanti-Kandarakis and Papavassiliou, 2006, Rajkhowa et al., 2009).

1.4. Contributing factors in insulin resistance

1.4.1. Obesity and Diet

Obesity is the single greatest risk factor for the development of insulin resistance and type 2 diabetes. This occurs when energy intake is greater than expenditure leading to increased adipose deposition. Men with a Body Mass Index (BMI) of 25 kg/m^2 have a relative risk of 2.2 for the development of type 2 diabetes compared to those with a BMI of 21 kg/m^2. This increases to a relative risk of 42.1 if they have a BMI of 35 kg/m^2 or over (Chan et al., 1994). The effect in women is even greater with a relative risk of 8.1 at a BMI of 25 kg/m^2 compared with 21 kg/m^2 rising to 93.2 at a BMI of greater than 35 kg/m^2 (Colditz et al., 1995) (Figure 1.3). The correlation of obesity with insulin resistance and type 2 diabetes is seen across all ethnic groups. However, this is an oversimplification of the problem. The distribution of body fat is also a determinant of insulin sensitivity with intra abdominal (visceral) fat more greatly associated with insulin resistance and type 2 diabetes than is subcutaneous fat (Kissebah and Krakower, 1994), meaning that many normal weight individuals have an increasing prevalence of insulin resistance particularly in ethnic groups, such as those from south Asia, who have proportionally more visceral fat than a Caucasian with the same waist circumference (Lear et al., 2007). The plasma and tissue levels of free fatty acids (FFA) can be increased by the consumption of a high fat diet and this contributes to hepatic insulin resistance (Boden et al., 1994, Samuel et al., 2004). With insulin resistance lipolysis is also increased due to a reduction in the inhibition of hormone-sensitive lipase. This increases FFA levels and further impedes insulin signalling leading to a

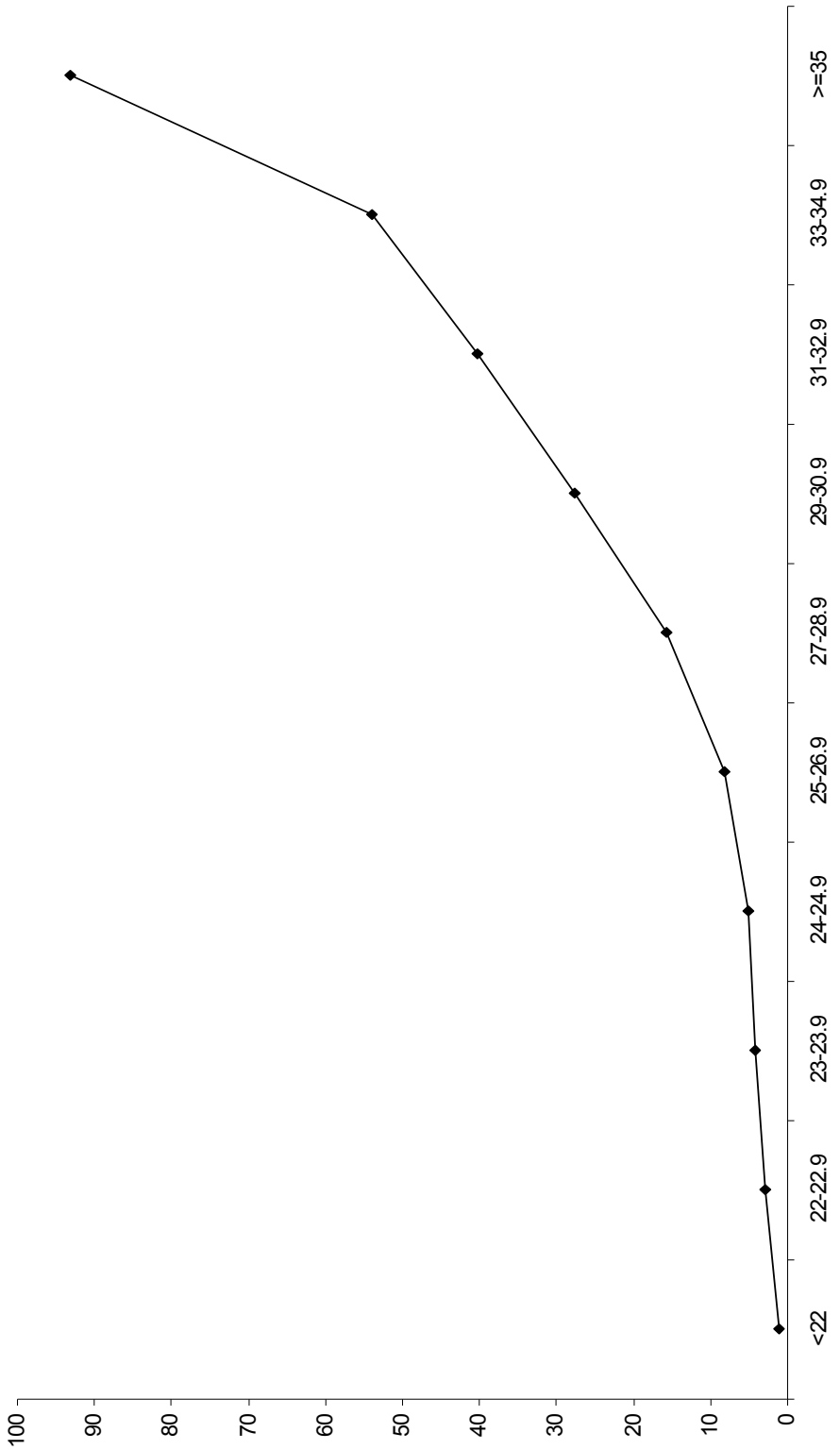


Figure 1.3 Age adjusted relative risk of development of type 2 diabetes in women at different BMI ranges (Colditz et al., 1995)

vicious cycle. Furthermore, lipogenesis is decreased in adipocytes due to a reduction in the activity of transcription factors such as peroxisome proliferator activated receptor (PPAR)γ (Guilherme et al., 2008). Free fatty acids can also induce inflammation in adipose tissue. Tissue macrophages are recruited to visceral adipose tissue where FFAs activate Toll-like receptors and promote the production of pro-inflammatory cytokines, tumour necrosis factor (TNF) α, interleukin (IL)-1β, IL-6 and monocyte chemotactic protein (MCP), via the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Shi et al., 2006). In the liver, TNFα induces insulin resistance by phosphorylation of IRS-1 at the inhibitory Ser307 site and also by increasing protein-Tyr phosphatase (PTP) 1-B production leading to the dephosphorylation of the active insulin receptor (Gao et al., 2002, Wu et al., 2011). Finally, a number of adipokines are disordered in obesity. In particular, the levels of adiponectin are lower and the leptin levels higher in the obese (probably generating leptin resistance) (Rabe et al., 2008). In leptin deficiency, exogenous leptin improves insulin sensitivity, but this does not occur in the presence of leptin resistance. Restoring levels of adiponectin can also enhance insulin sensitivity (Rabe et al., 2008). Therefore there are multiple potential molecular signals and pathways that could contribute to obesity induced insulin resistance and it remains unclear as to the relative importance of each, whether it is the same problem in all obese individuals or whether it is an accumulative effect of all of them that promotes insulin resistance in the obese population.

The effects of diet on insulin resistance are greater than just the effects of the ingestion of a hypercaloric diet. For example, saturated fat appears to be a potent inducer of insulin resistance, an effect that can be negated if there is a significant proportion of omega 3 fatty acid intake (Storlien et al., 1991, Storlien et al., 1987). On top of FFAs,

elevated triglycerides, which can occur as a result of high carbohydrate diets, independently can promote insulin resistance (Koyama et al., 1997). Fructose consumption in the form of high fructose corn syrup, leading to higher levels of triglycerides, saturated fat and also leptin resistance, is a further potential cause of insulin resistance (Thorburn et al., 1989, Hudgins, 2000, Shapiro et al., 2008).

1.4.2. Sedentary Lifestyle

Inactivity is an important risk factor for the development of insulin resistance and therefore exercise is associated with prevention of its development (Helmrich et al., 1991). Indeed, the higher the risk of an individual developing the disease, the more effective exercise becomes (Helmrich et al., 1991). There is a positive correlation between physical activity and insulin sensitivity (Mayer-Davis et al., 1998).

1.4.3. Other lifestyle factors

Cigarette smoking is known to decrease insulin action (Attvall et al., 1993). However the effects of alcohol and caffeine are less well defined (Keijzers et al., 2002, Petrie et al., 2004, Villegas et al., 2004, Kawamoto et al., 2009).

1.4.4. Genetic Factors in type 2 diabetes

The risk of developing type 2 diabetes is higher if one has a first degree relative with the condition. Indeed, the more relatives with type 2 diabetes an individual has, then the greater the risk. In twin studies monozygotic twins have been reported to have a concordance for the development of type 2 diabetes of between 34 and 100% (Barnett et al., 1981, Kaprio et al., 1992, Poulsen et al., 1999). The concordance rates for dizygotic twins are always lower suggesting that genetics plays a significant part in the

development of type 2 diabetes. More recently, modern gene mining techniques have identified a number of nucleotide polymorphisms that are associated with an increased risk of developing type 2 diabetes (including TCF7L2, PPARG, FTO, KCNJ11, NOTCH2, WFS1, CDKAL1, IGF2BP2, SLC30A8, JAZF1, and HHEX) (Lyssenko et al., 2008). At this time around 40 type 2 diabetes risk genes have been proposed, mostly associated with beta cell function but also linked to other aspects of glucose metabolism (Petrie et al., 2011). However, despite the large number of genes, each has a minor effect, detectable only in large population studies, and thus this information is unlikely to be useful in the clinic for early diagnosis. For example, those homozygous for the risk allele of TCF7L2 (7% of the study population) the most common mutation found, have approximately double the risk of developing type 2 diabetes (Grant et al., 2006). In addition, variations in FTO, the locus associated with differences in BMI, account for less than 0.5% of the overall genetic variance of the disease (Frayling et al., 2007). The less common variants have a lesser effect and as such an interaction between genetics and environment is the likeliest cause of insulin resistance.

1.5. Molecular Insulin Action

1.5.1. Tissue Specific Effects of Insulin

During fasting the levels of insulin are extremely low. After food intake, these rise rapidly to allow the metabolism and appropriate storage of ingested nutrients. Insulin sensitive tissues (liver, adipose and muscle) have common but also specialised responses to insulin stimulation (Table 1.1).

	Liver	Adipose	Muscle
Glucose Uptake		↑	↑
Glycogenesis	↑		↑
Glycogenolysis	↓		↓
Amino Acid uptake			↑
Protein Synthesis	↑	↑	↑
Protein Degradation	↓	↓	↓
Fatty Acid and Triglyceride Synthesis	↑	↑	
Lipolysis	↓	↓	
Lipoprotein Uptake		↑	
Cellular Respiration	↑	↑	↑

Table 1.1 Tissue specific effects of insulin

1.5.1.1. Liver

Unlike other insulin sensitive tissues, glucose uptake in liver is by passive diffusion and is not directly stimulated by insulin (El-Munshid et al., 2000) (although insulin stimulated glucose metabolism would indirectly increase passive diffusion into the hepatocyte). This is due to the fact that liver does not express the insulin sensitive glucose transporter (GLUT4), but rather GLUT2. Glucose in hepatic cells is rapidly converted to glucose-6-phospate (G6P) by glucokinase, an action opposed by glucose-6-phosphatase (G6Pase) which in turn is negatively regulated by insulin (Chapter 1.8 and 1.9.). The higher levels of glucose-6- phosphate induced by insulin can then be utilised in the glycolytic pathway. Furthermore, insulin increases the activity of glycogen synthase both by increasing G6P levels and by inhibiting the action of Glycogen Synthase Kinase 3 (GSK-3), an inhibitory kinase (Chapter 1.6.6). At the same time insulin turns off glycogen breakdown (Sindelar et al., 1996). Gluconeogenesis is the *de novo* production of glucose from non-carbohydrate precursors. During early stages of fasting this process is the major source of glucose and allows the maintenance of plasma glucose levels (vital to prevent hypoglycaemia). After feeding, and the subsequent availability of glucose, gluconeogenesis is not required. Insulin prevents gluconeogenesis in large part by repressing the production of the rate controlling enzymes in the process, phosphoenolpyruvate carboxykinase (PEPCK) and G6Pase (Chapter 1.9), and thus preventing *de novo* glucose formation.

Insulin also has effects on lipid metabolism in hepatocytes. Once glycogen stores are at maximal remaining glucose is shunted to the fatty acid synthesis pathway. Pyruvate is converted to Acetyl-CoA and then Malonyl-CoA by pyruvate dehydrogenase and Acetyl-CoA carboxylase (ACC) respectively, both of which are insulin sensitive (El-

Munshid et al., 2000). Using Acetyl-CoA and Malonyl-CoA as substrates, the fatty acid synthase (FAS) complex performs multiple condensation reactions to form fatty acids. FAS transcription is up regulated by sterol regulatory element binding protein-1c (SREBP-1c) (Chapter 1.10.5). These fatty acids are then combined with glycerol, also derived from glucose, to form triglycerides which are exported to adipose tissue through the blood as lipoproteins. Finally, insulin increases protein synthesis in many tissues including the liver by increasing general (El-Munshid et al., 2000) protein translation and also reducing proteolysis. (El-Munshid et al., 2000)

1.5.1.2. Adipose tissue

Lipogenesis occurs in adipocytes following feeding in response to insulin. Triglycerides absorbed from the intestine (chylomicrons), or those produced in the liver (lipoproteins), are hydrolysed to glycerol and free fatty acids by the action of lipoprotein lipase (LPL). Insulin induces LPL activity in adipocytes but also increases its secretion into the surrounding endothelium (El-Munshid et al., 2000). Fatty acids are taken up into the adipocytes by an insulin sensitive fatty acid transport protein (FATP1). Once in the adipocytes, free fatty acids must be combined with Co-enzyme A, another insulin induced process, before esterification to form triglycerides (El-Munshid et al., 2000). Glucose enters the adipocyte through the GLUT4 transporter and the number of GLUT4 molecules in the cell membrane is increased by insulin stimulating its translocation from internal stores. The glycerol required for triglyceride formation is formed from glucose within the adipocytes (El-Munshid et al., 2000). Furthermore, the breakdown of triglycerides is prevented by the dephosphorylation of hormone sensitive lipase which leaves this inactive (El-Munshid et al., 2000). Insulin also modifies the transcription and production of lipogenic genes through activation of PPARγ switching the adipocyte

proteome to one more suitable for adipogenesis. The effects on protein synthesis are the same as those seen in liver.

1.5.1.3. Muscle

The effects of insulin in muscle are similar to those seen in liver. Glucose uptake differs because in muscle this is an insulin dependent process requiring the translocation of GLUT 4 glucose transporters to the cell membrane. However, once the glucose has entered the myocyte, the process of increased glycogenesis, reduced glycogenolysis, increased cellular respiration and protein synthesis are similar to those seen in liver (El-Munshid et al., 2000). However, in contrast to liver, insulin increases amino acid uptake into skeletal muscle by increasing numbers of amino acid transporters in the cell membrane.

1.6. Insulin Signal Transduction (Figure 1.4)

1.6.1. Insulin Receptor

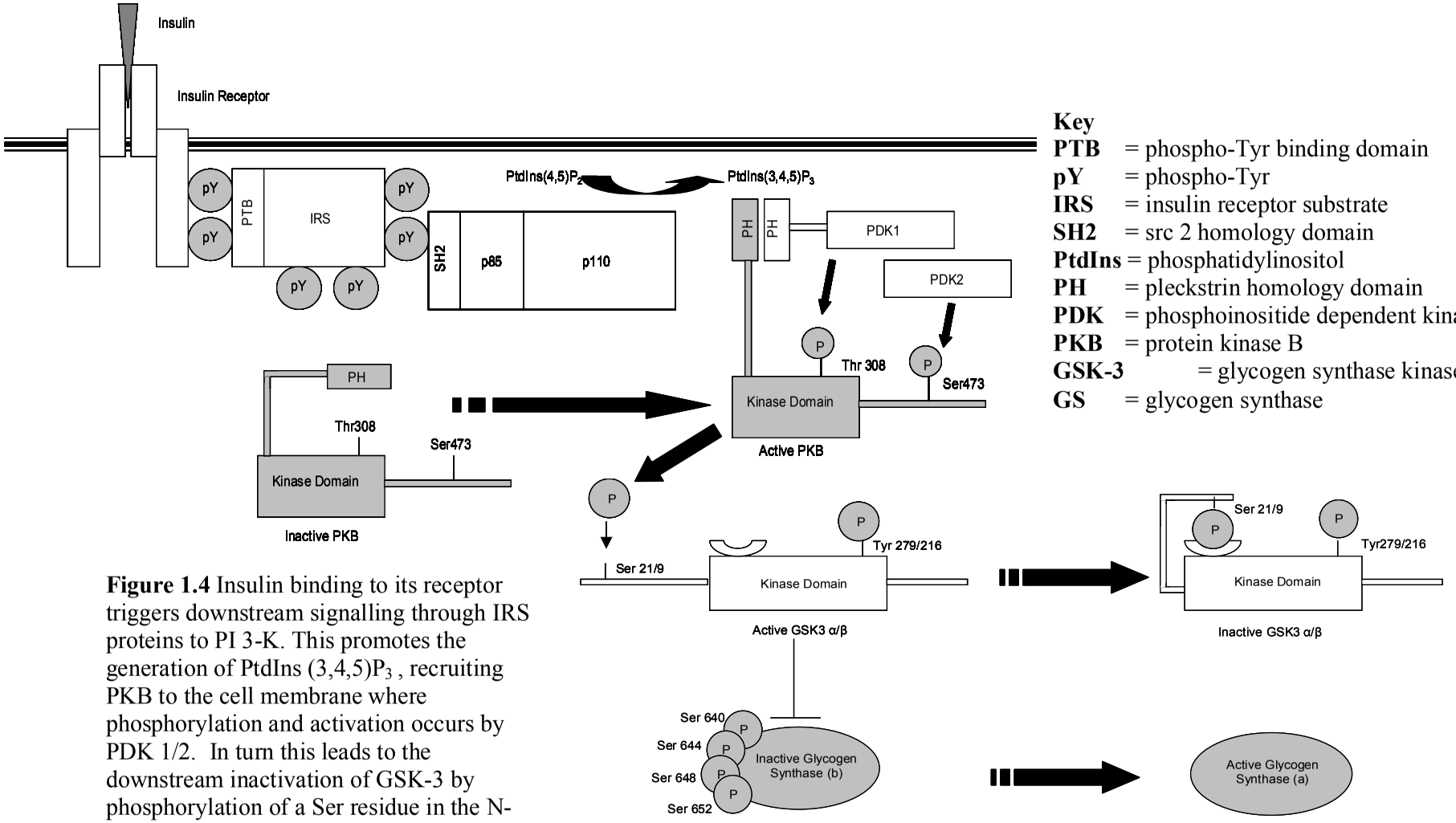
The insulin receptor is a heterodimeric Tyr kinase receptor consisting of 2 α (extracellular) and 2 β (transmembrane) subunits. The α subunit is entirely extracellular and contains the insulin binding motif. The β subunit contains a short extracellular region that includes the domain for interaction with the α subunit, a transmembrane domain and an intracellular region containing the Tyr kinase domain. On binding to insulin, the IR undergoes autophosphorylation which induces the intrinsic Tyr kinase activity of the receptor. The activated receptor recruits a number of IR target proteins (IRS, Gab-1, p60dok, Cbl, APS) of which the insulin receptor substrates (IRS) have gained the most interest (Pessin and Saltiel, 2000). The activated insulin receptor is

internalised and packaged in the endosome, where the insulin is dissociated and then degraded. The receptor is dephosphorylated and then recycled to the cell membrane.

1.6.2. Insulin Receptor Substrates (IRS).

There are 6 IRS molecules numbered 1-6, with distinct tissue distributions. IRS-1 and -2 are widely distributed, IRS-3 is mainly found in the brain and adipose tissue (rodents only), while IRS-4 is found only in embryonic tissues (Giovannone et al., 2000). There is little evidence for the involvement of IRS-5 and -6 in metabolic cell signalling (Cai et al., 2003). All of the IRS molecules have significant homology. They each have a pleckstrin-homology (PH) domain and a phospho-Tyr binding (PTB) domain close to the N-terminus which together mediate binding to the insulin receptor (IR), as well as other Tyr kinase receptors. In addition IRS contains a number of Tyr motifs, that when phosphorylated (e.g. by the IR) mediate binding to molecules containing src-homology (SH) 2 domains. Thus IRS proteins are often termed adaptor molecules as they link the insulin receptor to the downstream signalling cascade including key metabolic control pathways such as the phosphatidyl inositol (PI) 3-kinase.

However, insulin, as well as fatty acids and inflammatory mediators, also induces phosphorylation of a number of Ser residues of IRS molecules which, in most cases, negatively affect signalling, and if sustained, may contribute to insulin resistance. A number of insulin activated kinases (e.g. S6 Kinase, ERK, JNK) have been proposed to mediate the Ser phosphorylation of IRS and in the normal physiological setting may represent a feedback inhibition of insulin signalling in response to pulsatile hepatic insulin delivery (Harrington et al., 2004, Bouzakri et al., 2003, Aguirre et al., 2000). Sustained induction of phosphorylation of IRS at these sites would be expected to



Key

PTB = phospho-Tyr binding domain
pY = phospho-Tyr
IRS = insulin receptor substrate
SH2 = src 2 homology domain
PtdIns = phosphatidylinositol
PH = pleckstrin homology domain
PDK = phosphoinositide dependent kinase
PKB = protein kinase B
GSK-3 = glycogen synthase kinase 3
GS = glycogen synthase

Figure 1.4 Insulin binding to its receptor triggers downstream signalling through IRS proteins to PI 3-K. This promotes the generation of PtdIns $(3,4,5)P_3$, recruiting PKB to the cell membrane where phosphorylation and activation occurs by PDK 1/2. In turn this leads to the downstream inactivation of GSK-3 by phosphorylation of a Ser residue in the N-terminus .

reduce downstream signalling since two of the target residues, Ser 307 and 270, lie within the PTB domain and their phosphorylation leads to decreased binding to the insulin receptor (Aguirre et al., 2002, Craparo et al., 1997). Furthermore, the phosphorylation of IRS on Ser residues can also enhance ubiquitylation and targeting to the proteosome, hence hyperinsulinaemia leads to the degradation of IRS-1. Finally, insulin is reported to promote transcriptional inhibition of IRS-2 (Hirashima et al., 2003). A summary of IRS-1 phosphorylation sites is shown in Figure 1.5

The critical role of IRS molecules in the insulin signalling pathway is highlighted by gene knockout studies. Animals deficient in IRS-1 are viable but have significant intrauterine growth retardation. They are insulin resistant and glucose intolerant and develop other conditions associated with type 2 diabetes, namely hypertension and hypertryglyceridaemia. Interestingly, they do not develop overt diabetes due to a simultaneous increase in β-cell mass and hyperinsulinaemia (Abe et al., 1998, Araki et al., 1994, Tamemoto et al., 1994). The reconstitution of IRS-1 signalling by adenoviral construct infection can reverse these changes in IRS-1 null mice (Ueki et al., 2000). IRS-2 knockout mice have a different phenotype. They exhibit both hepatic and muscular insulin resistance along with a β-cell deficit and therefore overt diabetes. This leads to a hyperosmolar state and subsequent mortality (Withers et al., 1998, Kubota et al., 2000). Therefore, it is likely that as both isoforms are present in similar tissues, then both are required for normal insulin signalling. Dual knockout of IRS-1 and IRS-2 is embryonic lethal, but mice lacking both isoforms only in liver were viable and exhibited insulin resistance, glucose intolerance and hepatic steatosis (Taniguchi et al., 2005). A 70 -80% knockdown of IRS-1 led to increased expression of gluconeogenic enzymes and HNF- 4α, a decrease in glucokinase and a trend towards hyperglycaemia, whereas mice with a similar knockdown of IRS-2 upregulated SREBP-1c and FAS and therefore

had hepatic lipid accumulation. This suggests that there are different roles for each isoform, with IRS-1 involved mainly in glucose homeostasis and IRS-2 involved in lipid metabolism (Taniguchi et al., 2005). It is of note that in insulin receptor null hepatocytes IRS-1 phosphorylation in response to insulin is maintained, but phosphorylation of IRS-2 is not (Rother et al., 1998). In addition, liver specific knockout of IRS-2 on a global IRS-1 knockout produces a more profound effect on growth retardation and hyperglycaemia than IRS-1 -/- alone. Together these data suggest that IRS-2 is of particular importance in hepatic insulin signalling (Dong et al., 2006)

.

IRS-3 was originally isolated from rat adipocytes and shares approximately 50% homology with both the PTB and PH domains of IRS-1 and IRS-2 (Lavan et al., 1997b). In mice, mRNA for IRS-3 was found not only in adipocytes, but also in hepatic and lung tissue (Sciacchitano and Taylor, 1997). There is, so far, no evidence of a human IRS-3 homolog, and an in silico screen provides evidence against its presence (Bjornholm et al., 2002). Nevertheless, an IRS-3 knockout mouse model has been created. This displayed no altered phenotype in either growth or glucose homeostasis (Liu et al., 1999). However, mice with a dual knockout of IRS-1 and IRS-3 exhibit lipoatrophy, and overt hyperglycaemia associated with insulin resistance, B-cell hypertrophy, hyperinsulinaemia, but surprisingly no steatosis (Laustsen et al., 2002). These mice had decreased levels of leptin, the restoration of which improved their insulin sensitivity (Laustsen et al., 2002).

Likewise, IRS-4 was isolated from the Human Embryonic Kidney (HEK293) cell line (Lavan et al., 1997a). This also shared a significant homology of PTB and PH domains (approximately 40%) with IRS-1, 2 and 3 (Lavan et al., 1997a). Knockout of IRS-4

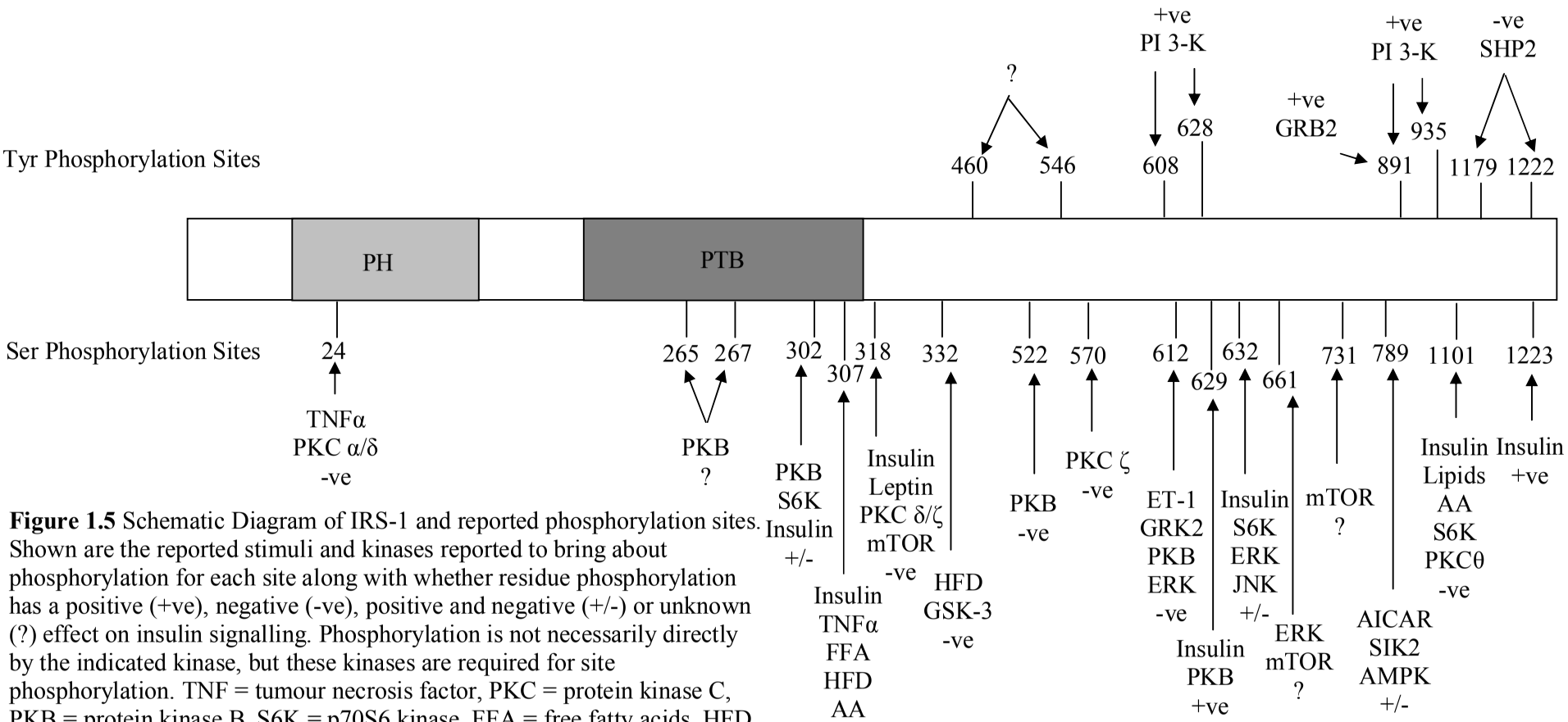


Figure 1.5 Schematic Diagram of IRS-1 and reported phosphorylation sites. Shown are the reported stimuli and kinases reported to bring about phosphorylation for each site along with whether residue phosphorylation has a positive (+ve), negative (-ve), positive and negative (+/-) or unknown (?) effect on insulin signalling. Phosphorylation is not necessarily directly by the indicated kinase, but these kinases are required for site phosphorylation. TNF = tumour necrosis factor, PKC = protein kinase C, PKB = protein kinase B, S6K = p70S6 kinase, FFA = free fatty acids, HFD = high fat diet, AA = amino acids, mTOR= mammalian target of rapamycin, JNK = c-Jun N terminal kinase, IKK = IκB kinase, GSK-3 = glycogen synthase kinase 3, ET-1 = endothelin-1, GRK2 = G protein–coupled receptor kinase 2, ERK = extracellular signal related kinase, AICAR = 5-Aminoimidazole-4-carboxamide riboside, SIK2= salt inducible kinase 2, AMPK = AMP activated protein kinase, PI 3-K = Phosphoinositide 3-kinase, GRB2 = Growth factor receptor-bound protein 2, SHP2 = SH2 domain-containing Tyr phosphatase-2. (Taniguchi et al., 2006, Boura-Halfon and Zick, 2009)

promotes a phenotype which includes reduced growth (male mice only), a reproductive deficit and an impaired response to an oral glucose tolerance test (Fantin et al., 2000)

1.6.3. Phosphoinositol 3-Kinase (PI 3-K)

PI 3-Ks are a group of lipid kinases made up of 3 classes (I, II and III). Upon activation they elicit downstream signalling pathways. PI 3-K phosphorylates the D3 position of the inositol ring of phosphotidylinositol (PtdIns) , PtdIns (4)P and PtdIns (4,5)P_2 thereby generating PtdIns (3)P, PtdIns (3,4)P2 and PtdIns (3,4,5)P_3 (PIP3) respectively. The latter two serve as intracellular messengers to propagate signalling pathways. Class I PI 3-Ks consist of a regulatory and a catalytic subunit and are further divided into Class Ia and Ib. Class Ia PI 3-K is a heterodimer containing one regulatory subunit (p85α, p85β, p55α, p55γ or p50α) and the catalytic p110 subunit (α, β or δ). Class Ib PI 3-Ks have a different regulatory subunit (p101 or p87) and catalytic subunit (p110γ). Class II PI 3-Ks consist solely of a catalytic subunit of which there are 3 currently known (PI 3-K-C2 α, PI 3-K-C2 β and PI 3-K-C2 γ). Only one class III PI 3-K has been identified (Vps34). This exists as a heterodimer with a regulatory subunit (p150) and is only able to convert PtdIns to PtdIns (3) P. There has been extensive study of the class I PI 3-Ks and their structure, function and role in intracellular signalling have been largely elucidated. Both p85α and β contain two SH2 domains at their C terminus, which allow binding to the IRS molecules along with other Tyr phosphorylated receptor kinases, but also have a breakpoint cluster domain and a SH3 domain at their N-terminus (Escobedo et al., 1991, Otsu et al., 1991). The p110 catalytic subunit binds to the inter SH2 domain with its N-terminal adaptor binding domain (ABD) (Klippel et al., 1993). The p110 subunit also contains a ras binding domain (RBD), a C2 domain which facilitates cell membrane binding, a helical domain and a catalytic domain at the C-terminus.

Knockout studies have not clearly elucidated the importance of this node in the insulin signalling pathway. A triple knockout of p85α-p55α-p50α is generally perinatal lethal, however survivors are more insulin sensitive. Similarly, mice heterozygous for the gene encoding PI 3-kinase alpha regulatory subunit (Gonzalez and McGraw, 2009), or mice lacking either p85α alone, p55α/p50α or p85β all display improved PI 3-K signalling (Fruman et al., 2000, Chen et al., 2004, Terauchi et al., 1999, Ueki et al., 2002, Mauvais-Jarvis et al., 2002). This increase in insulin sensitivity is surprising and remains unexplained but could suggest that these regulatory subunits are actually inhibitory. Consistent with this hypothesis the microinjection of a glutathione S-transferase-p85 fusion protein or a dominant-negative mutant of p85 prevents GLUT4 translocation (Haruta et al., 1995). Attempts to make a p110 knockout resulted in embryonic lethality (Bi et al., 1999). More information has been gained from the use of PI 3-K inhibitors which prevent many of the actions of insulin including glucose uptake in adipocytes (Okada et al., 1994), activation of PKB (Alessi et al., 1996) and repression of hepatic gluconeogenesis (Sutherland et al., 1995, Dickens et al., 1998). Furthermore, constitutively active mutants of PI 3-K are sufficient to mimic many actions of insulin, including stimulation of glucose uptake into adipocytes by translocation of GLUT4 to the cell membrane (Katagiri et al., 1996, Martin et al., 1996).

1.6.4. 3-Phosphoinositide Dependent Protein Kinase-1 (PDK1)

PDK1 is a master kinase of many AGC (cAMP-dependent, cGMP-dependent and protein kinase C) kinases most of which are involved in metabolism, cell survival and proliferation. The family includes, among others, PKB, p70S6K, SGK, RSK and atypical PKC (Alessi et al., 1997, Alessi et al., 1998, Kobayashi and Cohen, 1999, Frodin et al., 2000, Le Good et al., 1998). PDK-1 has an N-terminal kinase domain and a C-terminal PH domain which mediates the interaction with lipid second messengers

PtdIns (3,4)P_2 and PtdIns (3,4,5)P_3. PDK-1 does not require insulin stimulation for its activation but like all AGC kinases does require phosphorylation of its T-loop residue (Ser241) for activation (Casamayor et al., 1999). The ability of PDK-1 to autophosphorylate at this site means that the kinase is constitutively active. Therefore, the regulation of the phosphorylation of PKB by PDK-1 upon insulin stimulation is due to the co-localisation of these two kinases at the cell membrane via the interaction of their PH domains with lipid second messengers (Filippa et al., 2000, Watton and Downward, 1999, Anderson et al., 1998). PDK-1 also has a hydrophobic pocket in the catalytic domain that regulates activity and interaction with substrates (Biondi et al., 2000). The hydrophobic binding pocket interacts with a hydrophobic motif, PDK-1 interacting fragment (PIF), present in many of its substrates. The binding and phosphorylation of the hydrophobic motif may induce a conformational change in the substrate allowing further phosphorylation. This explains how substrates lacking a PH domain, e.g. S6K, SGK and RSK are regulated by PDK-1 (Biondi et al., 2001, Balendran et al., 1999).

Mouse models lacking PDK-1 are not viable (Lawlor et al., 2002). A 90% reduction in PDK-1 levels in all tissues produced animals which were small as a consequence of reduced cell size, but viable and fertile (Lawlor et al., 2002). Mutation of the PH domain of PDK-1 blocks its ability to activate PKB but leaves most of its other target phosphorylations intact. Mice expressing this mutant PDK-1 have small size and insulin resistance, emphasising the importance of the PDK-1-PKB interaction, although gluconeogenesis was still efficiently suppressed by feeding suggesting minimal PKB activity is required for this action of insulin (Bayascas et al., 2008).

1.6.5. Protein Kinase B

Protein Kinase B (PKB), also called AKT, is an AGC kinase, one of the major downstream targets of PDK-1 in the insulin signalling pathway and is instrumental in cellular processes involved in metabolism, cell proliferation and transcription (Gonzalez and McGraw, 2009). This protein contains an N terminal PH domain, a central Ser /Thr kinase domain and a C terminal regulatory domain containing a hydrophobic motif as with other AGC kinases.

There are 3 isoforms of PKB designated α, β and γ (AKT1, 2 and 3) (Coffer and Woodgett, 1991, Cheng et al., 1992, Brodbeck et al., 1999). There is differing tissue distribution of PKB isoforms. PKBα is ubiquitously expressed, but the lowest levels are found in pancreas and skeletal muscle, PKBβ is highly abundant in insulin responsive tissues and PKBγ although not highly expressed, is mostly found in brain and testis (Yang et al., 2003). Phosphorylation of PKBα at Thr308 and Ser473 is induced by insulin in a PI 3-K dependent manner (Kohn et al., 1995, Alessi et al., 1996) and all isoforms respond to PDK-1 in a similar fashion requiring phosphoinositides for this interaction (Walker et al., 1998). In the basal state, PKB is present in the cytoplasm, and the PH domain prevents phosphorylation. Upon insulin exposure, it is recruited to the cell membrane where the PH domain binds PIP3, inducing a conformational change that allows the phosphorylation of Thr308 by PDK1 (Filippa et al., 2000). The hydrophobic motif is phosphorylated at Ser473 by another kinase, most likely mTORC2 (Sarbassov et al., 2005). It can then interact with substrates, of which the two of the first characterised were GSK-3 and forkhead box O (FOXO).

As expected, mice lacking the PKBβ isoform exhibit hepatic insulin resistance, whereas those without the α isoform are small, but have normal glucose homeostasis (Cho et al.,

2001a, Cho et al., 2001b). In hepatocytes, inhibition of PKB (α + β) blocks insulin regulation of PEPCK and G6Pase (Logie et al., 2007). Immortalised adipocytes from PKBβ null mice have deficient glucose uptake and GLUT4 translocation and this can be rescued by the reintroduction of PKBβ, but not PKBα (Bae et al., 2003). The depletion of PKB by siRNA in mouse adipocytes reduces both PKB and phosphorylation of its substrate GSK-3 (Puri et al., 2007). In contrast, a constitutively active PKB increases glucose uptake in an adipose cell line (Kohn et al., 1996)

1.6.6. Glycogen Synthase Kinase 3 (GSK-3)

Glycogen Synthase Kinase 3 has 2 mammalian isoforms, GSK-3α (51 kDa) and GSK-3β (47 kDa) and is one of the kinases able to phosphorylate Glycogen Synthase (GS) (Woodgett and Cohen, 1984). The two isoforms have near identical kinase domains, but differ considerably in both the N- and C-termini (Woodgett, 1990). The phosphorylation of GS by GSK-3 occurs at 5 closely spaced Ser residues with the highly specific configuration SXXXS (P) (where X is any amino acid). For most GSK-3 substrates including GS, phosphorylation by another kinase (priming) is required before recognition of substrates by GSK-3 (Fiol et al., 1987, Picton et al., 1982, Rylatt et al., 1980). During basal conditions GSK-3 inhibits GS through phosphorylation of 4 Ser residues in the C-terminus, negatively regulating activity (Lawrence and Roach, 1997). On exposure of cells to insulin PKB phosphorylates GSK-3α/β at Ser21/9 (Cross et al., 1995) which inhibits its activity (Sutherland et al., 1993) and removes the inhibitory action it has on GS (Parker et al., 1983). The phosphorylation of Ser21/9 GSK-3 α/β causes the N-terminal domain of GSK-3 to move into a phosphate binding pocket of the kinase domain which is required for recognition of primed substrates (i.e. it disrupts substrate association by acting as a pseudosubstrate) (Frame et al., 2001).

In addition to the N-terminal inhibitory phosphorylation, there is a facilitative Tyr phosphorylation in the catalytic loop (Tyr279 for GSK-3α and Tyr216 GSK-3β). This site is constitutively phosphorylated due to autophosphorylation, thus leading to constitutively active GSK-3 (Cole et al., 2004). It is therefore unique among kinases being constitutively active in resting cells, inhibited by phosphorylation following cell stimulation, mostly requiring a primed substrate and generally being a negative regulator of cellular processes.

In addition to PKB regulating GSK-3 several other AGC kinases have been shown to phosphorylate the N-terminal site, at least *in vitro* (including p90RSK, p70S6K, PKC and PKA). Therefore, potentially, activation of the mitogen activated protein kinase (MAPK) and PI 3-K-mTOR pathways in cells could also promote phosphorylation of Ser21/9 (Sutherland and Cohen, 1994, Sutherland et al., 1993). However, inhibition of the mTOR pathway had no effect on the IGF-1/Insulin mediated phosphorylation of GSK-3 in L6 muscle cells, whereas inhibition of PI 3-K prevented the inactivation of GSK-3 (Cross et al., 1994). This strongly argues that insulin regulation of GSK-3 requires PKB activation, however Krebs and colleagues have reported a requirement for p42/44MAPK and p90RSK in the regulation of GSK-3 by growth factors (Eldar-Finkelman et al., 1995).

GSK-3β knockout mice die in utero due to hepatic degeneration and a conditional knockout of hepatic GSK-3β shows no metabolic phenotype (Hoeflich et al., 2000, Patel et al., 2008). In contrast, knocking out GSK-3β in skeletal muscles leaves animals more glucose tolerant (Patel et al., 2008). GSK-3α knock out animals display enhanced insulin and glucose sensitivity, reduced fat mass and increased hepatic glycogen content. Muscle glycogen levels are unaltered. These mice also exhibit relatively higher insulin

stimulation of PKB phosphorylation and higher levels of IRS-1 (MacAulay et al., 2007). In mice with an S9A GSK-3α, S21A GSK-3β or dual muscle knockin, only those with a mutation in β isoform showed a reduction in glycogen synthase activity (McManus et al., 2005). These genetic studies highlight the key role of GSK-3β in development, confirms the importance of both isoforms in glucose homeostasis and suggests isoform specific functions (although the molecular basis for this remains unclear).

1.6.7. Mitogen Associated Protein Kinase (MAPK)

The Mitogen Associated Protein Kinase (MAPK) family comprises a group of Ser /Thr kinases activated by a diverse range of extracellular stimuli to control mitosis,
cell proliferation or cell death. Mitogens (e.g. EGF, NGF, insulin and IGF-1), pro-inflammatory cytokines (e.g. interleukins) and other cellular stressors (bacterial lipopolysaccharide, heat shock and osmotic stress) all stimulate this pathway. This family has four main groups (ERK1/2, JNK, p38 MAPK and ERK5) which are all regulated through a related tiered phosphorylation cascade. Each MAPK is phosphorylated by a MAPKK (MAP Kinase Kinase) which in turn is itself phosphorylated by a MAPKKK (MAP Kinase Kinase Kinase). The ERK pathway (sometimes referred to as the classical MAPK pathway as it was the first to bear that name) is stimulated in response to insulin. This pathway consists of the MAPKKKs A-Raf, B-Raf, and Raf-1, the MAPKKs MEK1 and MEK2 (which are dual specificity kinases, phosphorylating both Tyr and Thr residues) and the MAPKs, ERK1 and ERK2. Upon insulin receptor activation, there is recruitment to the receptor/IRS complex of Grb2 (growth factor receptor-bound protein 2) which contains an SH2 domain (Pawson and Scott, 1997). Grb2 exists in a heterodimeric complex with SOS (son of sevenless), a guanine nucleotide exchange factor that releases GDP from Ras allowing the binding of GTP required for Ras stimulation. This promotes the interaction of Ras with Raf and

activation of the subsequent phosphorylation cascade. Raf phosphorylates MEK at Ser217 and Ser221 resulting in the phosphorylation of MAPK on a specific TEY motif in the activation loop of the kinase domain (Ray and Sturgill, 1988, Alessi et al., 1994, Payne et al., 1991). In contrast to the MAPKKKs and MAPKKs the MAPKs have a large number of substrates with a diverse range of actions (Roux and Blenis, 2004). Many of the substrates of ERK1/2 are involved in the control of gene expression and the subsequent regulation of cellular growth and metabolism (Roux and Blenis, 2004). ERK1 knockout mice display, among other changes, decreased adiposity and resistance to obesity and insulin resistance in response to high fat feeding (Bost et al., 2005). Knocking out ERK2 is embryologically lethal (Saba-El-Leil et al., 2003). Again the basis of these apparent isoform specific phenotypes remains unclear but it appears the ERKs are strong candidates for co-ordinating growth responses with metabolic status of cells. Interestingly, abnormal regulation of ERK by insulin in human muscle is associated with insulin resistance in women with PCOS (Rajkhowa et al., 2009).

1.7. Theory of Molecular Insulin Resistance

The appearance of insulin resistance *in vivo* coincides with poorer responses of tissues to given concentrations of exogenous insulin (e.g. during clamps). There is also little evidence of loss of insulin receptors on target tissues in insulin resistant individuals. It is therefore assumed that a defect in post-receptor signalling exists in one or more tissues in these individuals (Marshall and Olefsky, 1980). Therefore, research focus on insulin resistance has moved to identification of the initial cause (lifestyle or genetic) and initial site (which signalling pathway/molecule) of post-receptor defects.

Mouse genetic studies have suggested that the IRS proteins are required for almost all of insulins actions and as such a deficit at this level would seriously impair cellular insulin

sensitivity. Phosphorylation of IRS-1 at Ser307 is known to negatively regulate insulin signalling (Aguirre et al., 2002). Furthermore, phosphorylation of IRS-1 at Ser612 and 632, close to the SH2 domain, reduces the ability of the p85 regulatory subunit of PI 3-K to bind to IRS-1 (Gual et al., 2003). These are mediated through activation of, among others, the insulin sensitive MAPK and mTOR pathways. *In vivo* work shows that these mechanisms may be involved in the development of insulin resistance. The culture of primary myotubes from type 2 patients show a decrease in PI 3-K activity due to increased Ser632 IRS-1 phosphorylation (Bouzakri et al., 2003). Furthermore, *in vitro* muscle biopsies from lean controls, obese non-diabetics and obese diabetics show an inverse correlation between insulin sensitivity and expression of PI 3-K, Ser307 IRS-1 phosphorylation and PKC (protein Kinase C) (Bandyopadhyay et al., 2005). PKC, which can also be activated by free fatty acids, may have an important role in insulin resistance at the level of IRS-1. The θ isoform of PKC induces ser1101 IRS-1 phosphorylation and a constitutively active PKCθ reduces phosphorylation of PKB (Li et al., 2004). In addition, PKCα is responsible for Ser24 IRS-1 phosphorylation. This is in proximity to the PH domain and may disrupt binding to lipid second messengers (Nawaratne et al., 2006). Phosphorylation of IRS-1 at ser522 also negatively affects insulin signalling. PKB is required for phosphorylation at this site (Giraud et al., 2007). However, another PKB dependent phosphorylation site (Ser629) has been shown to positively regulate insulin signalling by interfering with phosphorylation of Ser636/639 by ERK (Luo et al., 2007). Finally, adipocytes from patients with diabetes showed a reduced ability of insulin to phosphorylate ser312 IRS-1 (Danielsson et al., 2005). The impairment of IRS-1 signalling in response to insulin may, in normal individuals, be responsible for the negative feedback response to allow the correct length and amplitude of response to insulin.

Muscles from obese diabetics have been shown to have lower PI 3-K activity, yet despite this, there is normal activation of PKB (Kim et al., 1999b). However, glucose transport into muscle remains impaired (Krook et al., 2000). So although there are changes in PI 3-K and this may explain some of the insulin resistance, it is likely that other deficits are present. Opposing the actions of PI 3-K is PTEN, and muscle specific deletion of this protects against insulin resistance (Wijesekara et al., 2005). However, polymorphisms of PTEN have no effect on the risk of developing type 2 diabetes (Hansen et al., 2001).

PKB has received major interest as the focus of molecular insulin resistance. The comparison of myotubes from controls and type 2 diabetics show site and isoform specific changes in PKB phosphorylation. A reduction in Ser473 phosphorylation of total PKB was observed, yet the phosphorylation of Thr308 was the same between the two groups (Cozzone et al., 2008). In contrast there was a reduction in Thr308 phosphorylation of PKBα, but no change in Ser473 phosphorylation, while the reverse was true for PKBβ (Cozzone et al., 2008). This suggests that PDK-1 activity is maintained in type 2 diabetes, but that either the effects of mTORC2 are diminished or that PHLPP1, reportedly specific for PKBβ Ser473 dephosphorylation and whose mRNA is upregulated in type 2 diabetes, promotes dephosphorylation of PKBβ but not PKBα (Cozzone et al., 2008). Furthermore, patients with type 2 diabetes exhibit higher levels of GSK-3 in muscle and correlates with a decreased GS activity (Nikoulina et al., 2000). Furthermore, the phosphorylation of GSK-3 by PKB is reduced in the skeletal muscle of those with type 2 diabetes (Krook et al., 1998).

In summary, there is evidence for defective insulin signalling in muscle from insulin resistant subjects, probably somewhere on the IRS-PKB pathway. However most of the

studies have been done in individuals with diabetes so these defects may not be initial problems rather a response to insulin resistance or hyperglycaemia or intervention. In addition most analysis is semi-quantitative and very difficult to perform in large numbers, so generally all studies are on small patient groups with little fine clinical phenotyping. The definitive answer to what is the initial post-receptor signalling defect that promotes clinical insulin resistance and whether it is the same in every insulin resistant individual (or in every tissue in the same individual) remains to be determined.

1.8. Hormonal Regulation of Hepatic Glucose Production

Gluconeogenesis is the *de novo* production of glucose from non-carbohydrate precursors (Figure 1.6). In the fasting state gluconeogenesis is responsible for approximately 80% of glucagon stimulated hepatic glucose output, but this falls to 50% in the fed state, with glycogenolysis making up the remainder (Beuers and Jungermann, 1990). This process occurs for the most part in the liver with lesser contributions from kidney and intestine. The effects of insulin on insulin sensitive tissues have already been discussed (Chapter 1.5.1). However, regulation of gluconeogenesis and hepatic glucose output is now discussed in more detail as it is a major contributor to insulin regulation of blood glucose and is defective in type 2 diabetes. Pyruvate is converted to oxaloacetate by pyruvate carboxylase in the mitochondria. Oxaloactetate is decarboxylated and phosphorylated by Phosphoenolpyruvate Carboxykinase (PEPCK), a rate controlling step in gluconeogenesis, to form phosphoenolpyruvate and GDP. Fructose-1,6-bisphosphate is generated from phosphoenolpyruvate. This is used to generate fructose-6-phosphate via the action of fructose-1,6-bisphosphatase. Fructose-6-phosphate is further transformed into glucose-6-phosphate. The dephosphorylation of G6P by glucose-6-phosphatase (G6Pase) is another rate-controlling step and is the final reaction in the production of glucose by both gluconeogenesis and glycogenolysis. The

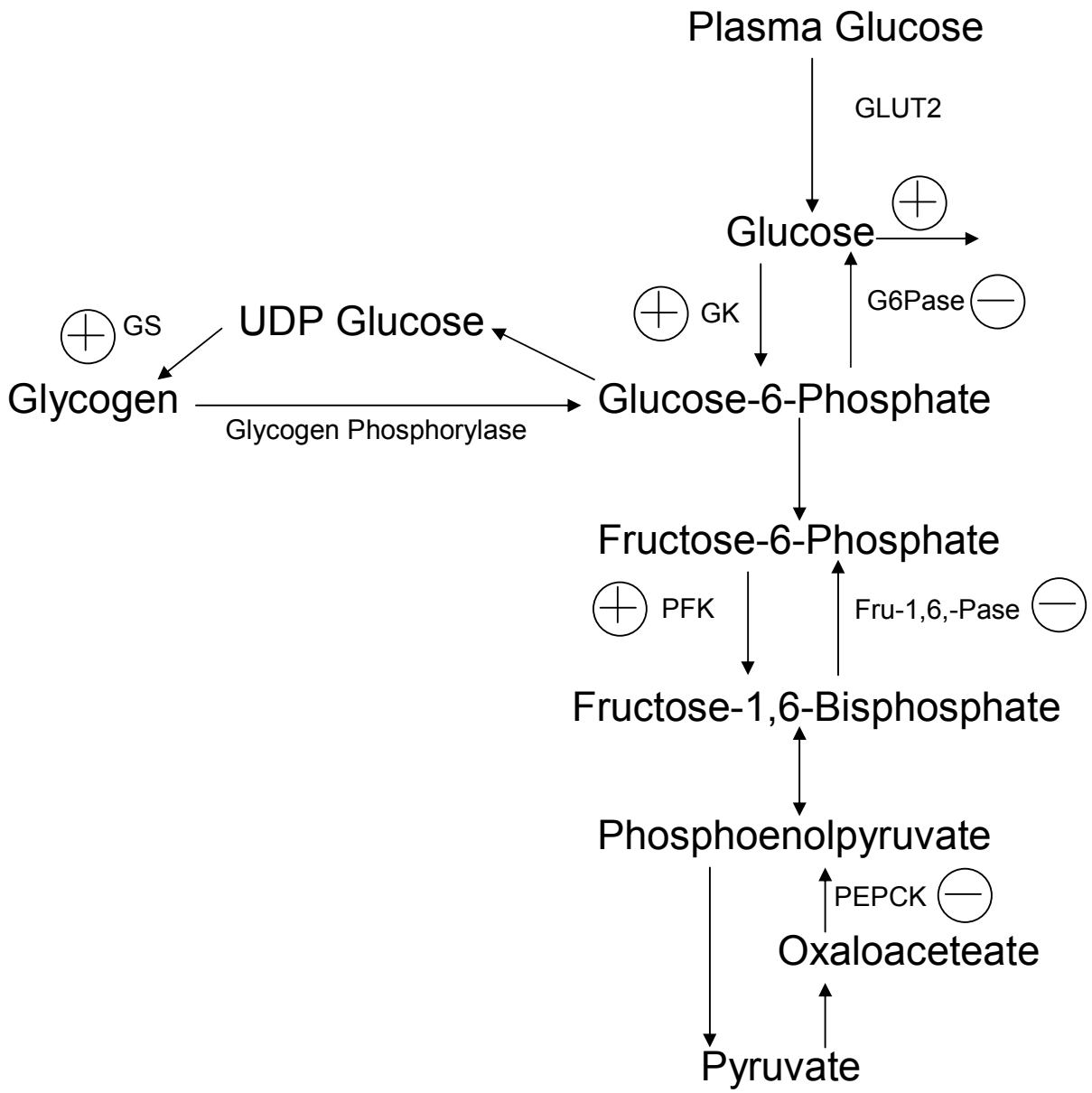


Figure 1.6 Schematic of gluconeogenesis in hepatocytes. + = stimulated by insulin, **-** = repressed by insulin, **GLUT2** = glucose transporter 2, **GK** = glucokinase, **G6Pase** = glucose-6-phosphatase, **GS** = glycogen synthase, **PFK** = phosphofructokinase, **Fru-1,6.-Pase** = Fructose-1,6,bisphosphatase, **PEPCK** = phosphoenolpyruvate carboxykinase,

role of PEPCK and G6Pase in producing excess glucose has come under intense scrutiny in the development of diabetes. Both of these genes are regulated by diet and hormones and as such may provide a link between lifestyle and the development of hyperglycaemia. The induction of hepatic glucose production, in intact liver, occurs almost immediately on exposure to glucagon and cAMP. It is thought that the immediate increases in hepatic glucose output occur through induction of glycogenolysis and inhibition of glycolysis rather than through changes in gene expression which have effects in the medium term (Lin and Accili, 2011). Indeed, insulin promotes glycogen synthase activity (Chapter 1.6) and this is opposed by PKA (a mainstay of glucagon signalling). Furthermore, glucagon activates fructose-1,6-bisphosphatase and inhibits pyruvate kinase driving the eventual formation of glucose-6-phosphate and insulin inhibits and activates both of these respectively. The hormonal control of gluconeogenesis by altering the rate of gene transcription has been known about for many years, with both cAMP and glucocorticoids stimulating and insulin suppressing this process (O'Brien and Granner, 1996). Underlying these changes are complex gene promoters.

1.9. Gene transcription

1.9.1. Phosphoenolpyruvate carboxykinase (PEPCK)

Phosphoenolpyruvate carboxykinase exists in two forms, cytosolic (cPEPCK) and mitochondrial (mPEPCK) which are transcribed from separate genes (Hanson and Reshef, 1997). Only the gene for the cytosolic isoform is regulated by diet and hormones, and from this point forward will be referred to as PEPCK. The tissue distribution of PEPCK is restricted mainly to liver, kidney and adipose tissue and to a lesser extent small intestine (Beale et al., 1985, Flores and Alleyne, 1971, Reshef et al., 1970, Anderson, 1970). The regulation of PEPCK has been extensively studied and is

induced by glucocorticoids, cAMP, triiodothyronine and retinoic acid (Hall et al., 1992, Lamers et al., 1982, Park et al., 1995), and all of these stimuli are dominantly repressed by insulin (Granner et al., 1983). Glucose also represses PEPCK independently of insulin, but only after metabolism by glucokinase (Cournarie et al., 1999, Scott et al., 1998). Conversely, lipids induce PEPCK transcription (Chen, 2007). The anatomy of the gene promoter and *cis*-acting elements has been mapped and a number of trans-acting factors are known to associate with the PEPCK gene promoter (Figure 1.7).

The PEPCK gene promoter can be divided into a basal regulatory unit, a cAMP response unit (CRU), a glucocorticoid response unit (GRU) and 2 accessory factor sites. The basal regulatory unit consists of the TATA box, a cAMP response element (CRE) and an NF-1 site. The CRE interacts with CREB and/or C/EBP (Faber et al., 1993). The CRU contains four C/EBP binding sites. Two separate CREs, CRE1 is a major target for PKA signalling whereas CRE2 is a weak C/EBP binding site, P3 and P4 sites (Liu et al., 1991, Roesler et al., 1989). Mutation of either CRE1 or P3 (I) completely abrogates the stimulation of gene expression by cAMP or PKA (Liu et al., 1991). An HNF-1 site is also present within the CRU. However, the deletion of this site has no effect on cAMP stimulated gene expression, but does reduce the basal expression of PEPCK (Liu et al., 1991, Yanuka-Kashles et al., 1994) (Figure 1.7).

The GRU contains two glucocorticoid response elements (GREs) and 3 Accessory Factor (AF-1, -2 and -3) binding sites and the two GREs, plus at least 2 out of 3 AFs are required for the induction of PEPCK by glucocorticoids (Imai et al., 1990). The AF1 site binds HNF-4 and COUP-TF along with the retinoic acid receptor (RAR) which mediates the stimulation of the gene promoter by retinoic acid (Hall et al., 1992, Hall et al., 1995). Within AF2 is the Thymine rich Insulin Response Element (TIRE) which

mediates the dominant repression of the gene by insulin and phorbol esters over cAMP and glucocorticoids (O'Brien et al., 1990). This site also binds a number of transcription factors (e.g. HNF-3, C/EBP and FOXO1) (O'Brien et al., 1995, Puigserver et al., 2003, Wang et al., 1996). The AF3 site binds COUP-TF along with the RAR (Scott et al., 1996). Furthermore, there is a thyroid hormone response element (TRE) which interacts with the C/EBP binding site in P3 (I) and mediates the induction by triiodothyronine (Hall et al., 1992, Park et al., 1995). Remote from the GRU, there are 2 distal accessory factor sites (dAFs). These allow enhanced expression of PEPCK in response to glucocorticoids by binding HNF-4 and PPAR nuclear factors (Cassuto et al., 2005).

The GRU alone does not explain the stimulation seen by glucocorticoids. In fact, the basal regulatory segment is required not only for promoter activity but also to synergise with the GRU for full stimulation of the PEPCK gene promoter (9 fold vs. 2 fold induction) (Imai et al., 1993). The CRE1 site enhances glucocorticoid stimulation by binding to CREB or C/EBPβ (Yamada et al., 1999), and the cross-talk between the GRU and CRU probably underlies the synergy between cAMP and glucocorticoid induction of the gene.

It was originally thought that insulin blocked the binding of accessory factors at AF2. However, as AF1, AF2, and both GREs are permanently occupied it is thought that hormonal stimulation causes binding factors to switch thus causing stimulation of the gene promoter (Faber et al., 1993). However, although it is known that insulin causes the rapid dissociation of multiple transcription factors from the PEPCK promoter finding the crucial factor involved in insulin suppression has not yet been possible (Hall et al., 2007). Also, FOXO1 is removed from the nucleus in response to insulin. It was thought that this may explain how insulin represses PEPCK transcription. However, at

normal cellular levels, the binding of FOXO1 to the AF2 region does not correlate with the response to insulin (Hall et al., 2000). This suggests that although FOXO1 is involved in the insulin mediated suppression of the PEPCK gene promoter, there is a requirement for other factors. However, it is known that insulin disrupts the FOXO1-PGC-1α complex potentially inhibiting the transcription of PEPCK (Puigserver et al., 2003). Finally, other methods of transcriptional regulation such as histone modification have been postulated (Hall et al., 2007).

1.9.2. Glucose-6-Phosphatase (G6Pase)

The regulation of G6Pase has much in common with PEPCK. Fasting, and hence glucocorticoids and cAMP, induce its expression and this is once again dominantly repressed by insulin (Mithieux et al., 1996). The anatomy of the gene promoter and *cis*-acting elements have been mapped (Figure 1.7), while a number of trans-acting factors are known to associate with the G6Pase gene promoter. The G6Pase gene promoter contains 2 CRE regions, 2 GREs, a TIRE, five putative FOXA (HNF3) binding sites, an HNF1, HNF4 and HNF6 binding site (Lin et al., 1997). Three of the FOXA binding sites are proximal and the other 2 more distal. The penultimate one of these overlaps the most proximal CRE (Lin et al., 1997). The most distal FOXA site overlaps the most distal GRE and the TIRE. Much more distally there are also binding sites for C/EBP and HNF4. CRE1 is required for the basal and cAMP induced stimulation of the gene promoter (Lin et al., 1997, Schmoll et al., 2000). Furthermore, as with PEPCK, it is also required for maximal stimulation of the gene by glucocorticoids (Schmoll et al., 2000). This site can bind C/EBPα, C/EBPβ and CREB. However, of these it seems as though only CREB can bring about transactivation (Lin et al., 1997).

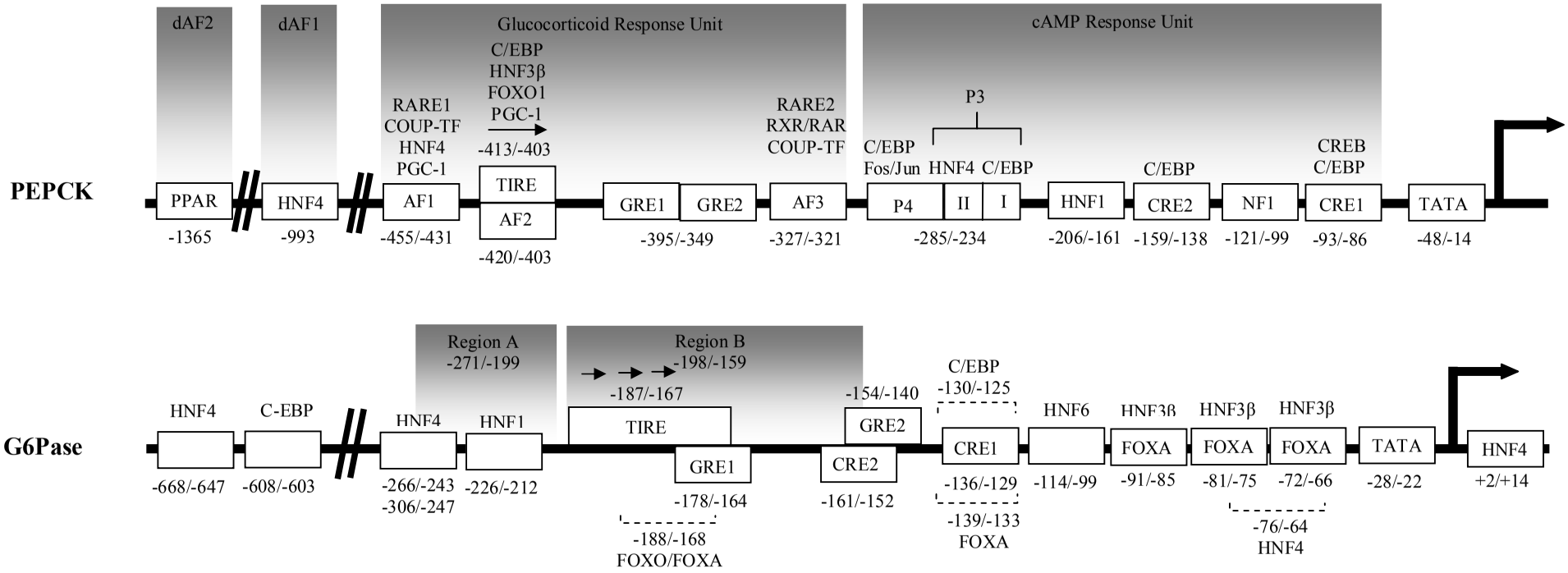


Figure 1.7 Structure of the PEPCK and G6Pase gene promoters. Regions of the promoter are shown as relative to the transcription start site. Dashed lines indicate putative binding sites. Arrows indicate direction of TIRE.
CRE = cAMP response element, **NF1** = nuclear factor 1, **HNF** = hepatocytes nuclear factor, **AF** = accessory factor binding site, **GRE** = glucocorticoid response element, **TIRE** = thymine rich insulin response element, **PPAR** = peroxisome proliferator-activated receptor, **CREB** = cAMP response element-binding protein, **C/EBP** = CCAAT-enhancer-binding proteins, **RARE** = retinoic acid response element, **TRE** =thyroid hormone response element, **RXR/RAR** = retinoic acid and retinoid x receptor, **COUP-TF** = chicken ovalbumin upstream promoter transcription factor 2, **FOXO/A** = Forkhead box O/A, **PGC-1** = Peroxisome proliferator-activated receptor gamma coactivator 1-alpha,

Stimulation of G6Pase gene expression by glucocorticoids involves the direct binding of the glucocorticoid receptor to the two GREs. However, as well as the CRE site previously mentioned, there is a requirement for intact HNF-1 and HNF-4 sites to allow complete promoter stimulation (Vander Kooi et al., 2005). Cyclic AMP stimulation of G6Pase gene expression requires the distal and proximal HNF4 sites and the distal C/EBP site (Gautier-Stein et al., 2005). These sites are not involved in basal expression of G6Pase. The final site shown to be involved in the cAMP induction is an HNF-6 site downstream of CRE1 (Streeper et al., 2001). Unlike PEPCK, G6Pase transcription is induced by glucose and lipids (Massillon et al., 1996, Massillon et al., 1997). The physiological role of this apparent paradox is unknown. The normalisation of glucose can reduce G6Pase gene expression, but only if the hyperglycaemia is not prolonged. However, these data suggest that both hyperglycaemia and hyperlipidaemia may, in the long term, lead to higher hepatic glucose output and diabetes, although the effects of insulin are dominant over those of glucose (Figure 1.7).

Basal gene expression is influenced by the two overlapping HNF-4 sites (-266/-243 and -306/-247) (Hirota et al., 2005) (Figure 1.7). The action of HNF-4 on the G6Pase promoter is synergistically enhanced by FOXO1 (Hirota et al., 2008). PGC-1 enhances the transcription of G6Pase through interaction with HNF-4 at a proximal binding site (-76/64) mutation of which destroys this interaction (Yoon et al., 2001, Boustead et al., 2003).

Insulin dominantly represses both basal and stimulated expression of G6Pase. Two regions, denoted A and B, are involved in this repression. The A region (-271/-199) overlaps an HNF-1 binding site (-226/-212) and the B region (-198/-159) contains 3 copies of the TIRE sequence (Streeper et al., 1997) (Figure 1.7). Interestingly, the

HNF-1 binding site in region A is required for full insulin mediated repression of gene expression, and as such is involved in both this and stimulation of the gene promoter (Streeper et al., 1998). One factor that interacts with the TIRE sequences of the B region is FOXO1. The binding of FOXO1 to this region correlates with the regulation of G6Pase gene transcription, but appears to involve only two of the TIRE sequences (named insulin response sequences originally, IRS-1 and IRS2) (Onuma et al., 2006, Ayala et al., 1999). Disruption of this interaction by nuclear exclusion of FOXO may be how gene transcription is down regulated by insulin. The role of the third TIRE is, as yet, unclear (Vander Kooi et al., 2003).

1.9.3. Insulin like growth factor binding protein -1 (IGFBP-1)

Although not involved in gluconeogenesis IGFBP-1 does indirectly affect insulin action through regulation of IGF-1 responses, while the regulation of IGFBP1 gene transcription has much in common with that of PEPCK and G6Pase. The promoter is stimulated by both cAMP and glucocorticoids and dominantly repressed by insulin (Powell et al., 1991, Suwanichkul et al., 1993). The promoter consists of a CRE, 2 GREs, 2 TIRE sequences and an HNF-1 binding site (Suwanichkul et al., 1993, Suh et al., 1994, Suwanichkul et al., 1994). As with the other gene promoters, cAMP induces gene transcription through the CRE (Suwanichkul et al., 1993). The GRE bind the GR weakly and full glucocorticoid stimulation requires an intact TIRE which acts as an accessory element for gene transcription (Suwanichkul et al., 1994). Furthermore, the HNF1 binding site acts as an accessory element for induction of IGFBP-1 gene promoter transcription (Suh and Rechler, 1997). The IGFBP1 TIRE is, as with the PEPCK and G6Pase gene promoters, required for the repression of gene transcription by insulin (Suh et al., 1994). Mainly by inference from signalling studies and sequence homology, FOXO1 has been implicated in this response.

1.10. Molecular aspects of insulin regulation of gene transcription

The effect insulin has on downstream gene promoters is through interaction of hormone regulated transcription factors.

1.10.1. Forkhead Box (FOXO) proteins

There are 19 classes of FOX proteins (A-S) of which FOXO contains four members (FOXO1, 3, 4 and 6). The *C. elegans* analog of FOXO1, daf-16, is genetically downstream of insulin signalling and so it was suggested that this transcription factor may be the link between the insulin receptor and suppression of gene transcription in mammalian cells (Ogg et al., 1997, Lin and Accili, 2011). FOXO proteins contain an N-Terminal forkhead DNA binding domain, a nuclear localisation signal, a nuclear export sequence (NES) and a C-terminal transactivation domain (Obsil and Obsilova, 2008). In the presence of cAMP FOXO1 is retained in the nucleus, where it interacts with the TIRE sequence of insulin regulated genes (PEPCK, G6Pase, IGFBP-1), and remains in a dephosphorylated state (Durham et al., 1999, Hall et al., 2000, Schmoll et al., 2000). In the presence of insulin, which behaves in a dominant manner, PKB is phosphorylated (Chapter 1.6.5). The now active PKB phosphorylates FOXO1 on Thr24, Ser256 and Ser319 (Rena et al., 1999). The phosphorylation of FOXO1 by PKB creates binding sites for 14-3-3 proteins (Thr24 and Ser256). This complex is moved to the cytoplasm and the 14-3-3 proteins prevent nuclear re-entry due to interference with the nuclear localisation signal, thus preventing interaction with its target genes (Zhao et al., 2004, Nakae et al., 2001). In addition, the Ser256 site lies in proximity to the DNA binding domain and phosphorylation reduces the DNA binding capacity of FOXO1 (Zhang et al., 2002). Within the cytoplasm, FOXO1 is targeted for degradation by the proteosome through ubiquitination (Matsuzaki et al., 2003).

Although it has been shown that FOXO1 binds to TIRE sequences, how it regulates gene transcription is still unknown. In fact, FOXO1 and its interaction with the TIRE does not account for the total effect of insulin on G6Pase gene expression (Schmoll et al., 2000). Furthermore, more than one of the FOXO family may interact with the G6Pase gene promoter to induce transcription. Knockout mice with a liver specific deletion in FOXO1 show similar fasted, but greatly reduced fed levels of G6Pase, PEPCK and IGFBP-1 (Matsumoto et al., 2007). Whereas a triple knockout of FOXO 1, 3a and 4 has no changes in the fasting levels of G6Pase or IGFBP-1, but a significant reduction in PEPCK mRNA (Haeusler et al., 2010). FOXO1 also has differential effects on different insulin responsive genes. For example, the overexpression of FOXO1 has been shown to reduce G6Pase mRNA, but not PEPCK. Conversely, the effect of a dominant negative FOXO1 mutant is to reduce cAMP and glucocorticoid stimulation of both PEPCK and G6Pase gene expression (Nakae et al., 2001). However, the decrease in the level of nuclear (unphosphorylated) FOXO1 correlates with the repression of both G6Pase and PEPCK mRNA in dogs subjected to hyperinsulinaemia (Ramnanan et al., 2010). Nevertheless, expression of a constitutively active FOXO1 increases basal levels of G6Pase and IGFBP-1 mRNA, and prevents the insulin suppression of both genes, yet there is no effect on PEPCK mRNA (Nakae et al., 2002). In contrast, PEPCK is transactivated by overexpression of FOXO3, as is IGFBP-1 and in both cases this can be inhibited by insulin, although endogenous FOXO3 doesn't appear to bind to these gene promoters (Hall et al., 2000). Therefore, although FOXO1 binds to TIRE sequences, it remains unclear if it is the only or even the major mediator of insulin regulation of any or all of these 3 genes.

1.10.2. Peroxisome Proliferator-Activated Receptor γ Coactivator-1α (PGC-1α)

Peroxisome Proliferator-Activated Receptor γ Coactivator-1α (PGC-1α) is a transcription factor that promotes transcription of gluconeogenic genes in the fasting state (Yoon et al., 2001). Levels of PGC-1α in the liver of models of type 2 diabetes have been shown to be elevated (Yoon et al., 2001). In primary hepatocyte cultures glucocorticoids and cAMP also induce PGC-1α production (Yoon et al., 2001). For full activation of the PEPCK gene promoter co-activation is required between PGC-1α and the GR and HNF4α (Yoon et al., 2001). Therefore, this transcription factor appears to be at the centre of fasting induced gluconeogenic gene expression. Furthermore, PGC-1α requires the presence of FOXO1 to promote transcription (Matsumoto et al., 2007). However, PGC-1α knockouts have similar fasting levels of PEPCK and G6Pase to wild type animals and in the fed state knockout animals fail to suppress PEPCK and G6Pase levels. This suggests that PGC-1α is essential for the appropriate response to nutritional regulation or at least for proper expression of the transcription factors required for nutritional regulation, although because PGC-1α is such an important transcription factor, it must be remembered that these animals probably lack a significant number of other transcription factors. (Lin et al., 2004). However, whole body overexpression of PGC-1α leads to hepatic insulin resistance secondary to higher levels of gluconeogenic genes, but increased muscle sensitivity is also reported (Liang et al., 2009).

1.10.3. cAMP Response Element Binding Protein (CREB)

CREB is a transcription factor that although discovered nearly 20 years ago (Montminy and Bilezikjian, 1987) is as yet incompletely understood. It acts as a scaffold allowing the interaction of other transcription factors (CBP, p300, TORC2) with gene promoters containing a CRE, thus inducing transcription (Koo et al., 2005). It is activated by PKA, via Ser133 phosphorylation which enhances interaction with TORC2 through the CREB

bZIP domain (Conkright et al., 2003, Gonzalez and Montminy, 1989). However, both insulin and glucagon lead to phosphorylation of the same site on CREB (Koo et al., 2005, Gonzalez and Montminy, 1989, Klemm et al., 1998) leaving us unsure how fasting and feeding regulate this transcription factor. Interestingly, GSK-3 can phosphorylate CREB at Ser129, after priming by PKA at Ser133 (Fiol et al., 1994), suggesting that insulin would reduce Ser129 phosphorylation although this remains to be validated. What is known is that a dominant negative CREB leads to fasting hypoglycaemia which can be reversed by overexpressing PGC-1α (Herzig et al., 2001).

1.10.4. Transducer of Regulated CREB Activity 2 (TORC2)

Transducer of regulated CREB activity 2 (TORC2), also called CRTC2 is a transcriptional coactivator that enhances CRE dependent gene transcription by interacting with the bZIP domain of CREB (Conkright et al., 2003). In the dephosphorylated state TORC2 translocates to the nucleus with PKA where it recruits CBP, p300 and CREB, activates the complex and promotes transcription of PEPCK and G6Pase (Koo et al., 2005). Levels of TORC2 are elevated in the livers of mouse models of obesity (Dentin et al., 2007). Liver specific and whole body knockout of TORC2 both show reduced hepatic gluconeogenic gene expression (Le Lay et al., 2009, Wang et al., 2010). SIK2 is a substrate of PKB and as such can be considered as part of the “classical” insulin signalling pathway (Dentin et al., 2007). Once phosphorylated by PKB at Ser358 it then phosphorylates TORC2 at Ser171. This leads to the translocation of TORC2 to the cytoplasm where it is targeted for ubiquitylation and proteasomal degradation (Dentin et al., 2007). This residue can also be phosphorylated by AMPK (Koo et al., 2005). Hence if TORC2 is part of the cAMP or glucocorticoid pathway to the PEPCK and G6Pase gene promoters then this PI 3-K-PKB-SIK pathway could explain the dominant repressive effect of insulin of these promoters. However this

remains to be conclusively proven as a key mediator of control of PEPCK and G6Pase gene transcription.

1.10.5. Sterol Regulatory Element Binding Protein-1c (SREBP-1c)

There are 3 members of the sterol regulatory element binding proteins (SREBP-1a, -1c and -2). They are transcription factors of the helix-loop-helix family, and their maturation is tightly controlled by the level of cholesterol in membrane. Therefore, they are responsible for cholesterol biosynthesis, but SREBP-1c also has a major role in fatty acid metabolism by inducing the transcription of FAS thereby promoting fatty acid synthesis (Desvergne et al., 2006). They also play a role in insulin regulated gene transcription. Indeed, the levels of SREBP-1c are increased by a high carbohydrate meal after fasting (Horton et al., 1998). Insulin, in a PI 3-K and PKB dependent manner increases the level of SREBP-1c (Fleischmann and Iynedjian, 2000), while overexpressing SREBP-1c leads to a reduction in the expression of PEPCK both *in vitro* and *in vivo*, without altering the levels of G6Pase mRNA (Foufelle and Ferre, 2002, Chakravarty et al., 2001, Becard et al., 2001). This suggests that SREBP-1c (and possibly other members of the family) may provide co-ordinated regulation of sterol/fatty acid synthesis and glucose synthesis, at least in the liver.

1.10.6. Small Heterodimer Partner (SHP)

SHP is an orphan nuclear hormone receptor that is most highly expressed in the liver and is best known for repressing gene transcription (Seol et al., 1996). It contains ligand binding and dimerization domains, but lacks a DNA binding domain (Seol et al., 1996). It can repress gene expression by inhibiting the transactivation ability of the retinoic acid receptor (RARα), retinoid X receptor (RXRα) or the thyroid receptor (TR) to which it binds (Seol et al., 1996). In addition, SHP inhibits the DNA binding of HNF-3α, β and

γ and HNF4 (Kim et al., 2004, Seol et al., 1997). Furthermore, SHP may antagonise the stimulatory effect of PGC1α on gene transcription and inhibit the DNA binding of CEBPα and the GR (Borgius et al., 2002, Park et al., 2007, Wang et al., 2005). SHP suppresses gluconeogenic gene expression in response to bile acids through the inhibition of both HNF4 and FOXO1 (Yamagata et al., 2004).

1.11. Intracellular signalling control of gluconeogenic genes

The insulin signalling pathway (Chapter 1.6) that links the insulin receptor to gluconeogenic genes has been extensively studied (Chakravarty et al., 2005, Barthel and Schmoll, 2003, Mounier and Posner, 2006). Mouse liver specific knockouts of both IRS-1 and –2 lose the inhibitory effect of insulin on PEPCK, G6Pase and IGFBP-1, and this phenotype can be rescued by the liver specific knock out of FOXO1 (Dong et al., 2008). Furthermore, blockade of the insulin signalling pathway at different levels yield similar results. The repression of PEPCK, G6Pase and IGFBP-1 is both PKB and PI 3-K dependent as shown in studies with small molecule inhibitors (Logie et al., 2007, Gabbay et al., 1996, Patel et al., 2002, Band and Posner, 1997). Overexpression of the catalytic p110 subunit of PI 3-K is sufficient to suppress PEPCK and G6Pase transcription, whereas expression of a dominant negative mutant of the PI 3-kinase regulatory p85α subunit is sufficient to increase transcription of the same genes (Miyake et al., 2002, Dickens et al., 1998, Kotani et al., 1999). In addition, PEPCK, G6Pase and IGFBP-1 transcription can be repressed by a constitutively active PKB (Cichy et al., 1998, Ono et al., 2003, Kotani et al., 1999). However, expression of a dominant negative form of PKB does not prevent the insulin induced repression of PEPCK or G6Pase transcription but does reduce regulation of IGFBP-1 (Cichy et al., 1998, Kotani et al., 1999, Dickens et al., 1998). Meanwhile, inhibition of mTOR by rapamycin prevents insulin repression of IGFBP-1 but has no effect on PEPCK or G6Pase (Patel et

al., 2002). This is only required for acute regulation of IGFBP1 as 24 hour inhibition with insulin is not rapamycin sensitive (Finlay et al., 2006). The role of GSK-3 in the regulation of all 3 genes is similar. The overexpression of GSK-3 has no effect on basal or insulin regulated levels of PEPCK or G6Pase but does increase levels of IGFBP-1 (Lochhead et al., 2001, Finlay et al., 2004). However, inhibition of GSK-3 with a number of different classes of inhibitors mimics the effects of insulin by repressing all 3 genes (Finlay et al., 2004, Lochhead et al., 2001). In contrast, mice expressing an insulin-insensitive GSK-3 do not have altered regulation of any of the genes in response to feeding (Lipina et al., 2005), suggesting that feeding does not need to inhibit GSK-3 as part of its mechanism to turn the genes off, yet GSK-3 activity is required for activity of all 3 promoters. In summary, although there are common elements between the promoters, the differential regulation suggests that additional insulin signalling pathways other than PI 3-K/PKB/GSK-3 can mediate at least part of the insulin regulation of gene transcription and there may be a level of redundancy. It is likely that other transcription factors and regulatory elements remain to be discovered to account for these differences.

There is evidence that other signalling pathways can influence insulin regulated gene transcription. Salt inducible kinases (SIK1 and 2) indirectly affect the stimulation of gluconeogenic genes. Under fasting conditions in mice, or under the effects of glucagon in primary hepatocytes, SIK1 levels were increased correlating with a decrease in the levels of PEPCK and G6Pase mRNA. This may be related to SIK regulation of TORC2 which is discussed earlier. This is thought to be part of a negative feedback loop to contain gluconeogenesis (Koo et al., 2005).

In addition modulation of AMP activated protein kinase (AMPK) may also influence the transcription of the PEPCK and G6Pase genes. A large part of the maintenance of cellular energy homeostasis occurs through the AMPK, an enzyme that is activated by physiological increases in AMP (Carling, 2004, Hardie, 2011). In times of energy deficiency, when AMP levels rise, the activation of AMPK appears central to stopping anabolic processes and shifting to catabolic processes to normalise ATP levels. The structure is a heterotrimeric complex consisting of a catalytic α subunit and two regulatory subunits (β and γ). There are 2 α isoforms (α1 and α2), 2 β isoforms (β1 and β2) and 3 γ isoforms (γ1, γ2, and γ3). The α1 subunit is expressed in most tissues, whereas the α2 subunit is mainly expressed in the liver, skeletal muscle and heart. The α subunit has an N-terminal catalytic domain, an autoinhibitory domain and a subunit binding domain at the C-terminus. The catalytic domain contains the Thr 172 T-loop phosphorylation site which is required for kinase activation (Hawley et al., 1996). The γ subunits contain 4 cystathione-β-synthase (CBS) domains which allow rapid sensing of changes in the AMP:ATP ratio. Each CBS domain can bind a single molecule of AMP (or ATP albeit with a much lower affinity), which increases the binding affinity of AMP to the next CBS domain (Carling, 2004, Adams et al., 2004). The binding of AMP induces a conformational change which exposes the α-subunit phosphorylation site allowing phosphorylation by an upstream kinase. AMP binding also inhibits the dephosphorylation by PP2A and PP2C along with allosteric activation of the phosphorylated kinase (Davies et al., 1995, Carling et al., 1989). Due to the fact that the α2 subunit is mostly present in insulin sensitive tissues, mice lacking this subunit are glucose intolerant, with a β-cell defect and skeletal muscle insulin resistance (Viollet et al., 2003). The currently validated upstream kinases are LKB1 and Calcium/calmodulin-dependent protein kinase kinase (CAMKK). LKB1 exists in a heterotrimeric complex with STRAD and MO25, and is a master kinase of a number of AMPK –like kinases

(Lizcano et al., 2004). Particularly in neurons, CAMKK is responsible for the phosphorylation of AMPK in response to calcium flux (Hawley et al., 2005).

The activity of the majority of AMPK in the liver appears to be under the control of LKB1, such that adult mice homozygous for LKB1 deletion in the liver display almost complete loss of AMPK activity. Furthermore, they also show decreased TORC2 phosphorylation and subsequent upregulation of PGC1-α which is associated with enhanced hepatic glucose output. This phenotype could be rescued by the addition of shRNA against TORC2 (Shaw et al., 2005).

The regulation of gluconeogenic gene expression by AMPK has mostly been performed in studies examining metformin action. Metformin treatment of liver cells (in culture or *in vivo*) reduces PEPCK and G6Pase gene expression (Kim et al., 2008, Foretz et al., 2010). It has been proposed that metformin activates AMPK (Zhou et al., 2001), which then activates SHP or inhibits TORC2 (Koo et al., 2005, Kim et al., 2008). For example, AMPK phosphorylates TORC2 at the Ser171 site *in vitro*, a not entirely surprising discovery as SIK2 is a member of the AMPK superfamily (Koo et al., 2005). AICAR, a molecule that mimics AMP has been shown to induce TORC2 phosphorylation in hepatocytes (Koo et al., 2005). However, direct activation of AMPK in cells by A-769662 does not increase the phosphorylation of TORC2 which suggests that AMPK doesn't directly regulate TORC2 in cells (Foretz et al., 2010). However, these differences may be more representative of the differences in the mechanism of action of the compounds.

Finally, expression of a constitutively active AMPK induces the transcription of SHP while a dominant negative AMPK does the converse (Kim et al., 2008). However,

changes in gluconeogenic gene transcription occur before changes in SHP gene transcription, which suggests that acute regulation of these genes by AMPK (and potentially metformin) is not through induction of SHP but it remains possible that SHP could mediate regulation in response to more chronic stimulation (Kim et al., 2008).

1.12. Therapeutic interventions for Insulin Resistance

1.12.1. Lifestyle

Lifestyle modification is at the heart of the treatment of insulin resistance. It is also important for the prevention of the sequelae of diabetes. The cessation of smoking is of vast importance, not only due to its effects on insulin resistance, but also for the prevention of associated cardiovascular disease. The effects of exercise on the treatment of insulin resistance are great. For example, in those with impaired glucose tolerance, there is a relative risk reduction of between 46% and 58% in the development of type 2 diabetes over 3-6 years with improved exercise (Tuomilehto et al., 2001, Lindstrom et al., 2003, Li et al., 2008, Knowler et al., 2002). Even in those with diabetes, exercise improved glycaemia and triglyceride levels whether performed in a structured or unstructured format suggesting an improvement in insulin sensitivity (Thomas et al., 2006). Dietary intervention can also have large effects. Extensive calorie restriction (600Kcal/day) in those newly diagnosed with diabetes can normalise hepatic insulin sensitivity (Lim et al., 2011). Further evidence of the effects of dietary restriction on insulin sensitivity comes from trials of bariatric surgery (Levy et al., 2007). However, some of the effects of this will be mediated by lower levels of obesity.

1.12.2. Current Insulin Sensitising Drugs

1.12.2.1. Metformin

Metformin is currently the only Biguanide class of drug used in clinical practice. Its use leads to increased insulin sensitivity and a reduction in hepatic glucose output, but its mechanism of action remains a matter of intense study. In rats treated with metformin there are lower levels of SREBP-1 in the liver which may lead to lower gluconeogenic gene expression (Zhou et al., 2001). In addition, metformin has been shown to disrupt the interaction of CREB with TORC2 leading to a reduction in gluconeogenic gene expression (He et al., 2009, Le Lay et al., 2009). In liver from LKB-1 knockout mice, the action of metformin on gluconeogenesis is ablated, however it is preserved in liver from AMPK knockout mice suggesting that the action of metformin is downstream of LKB1, but not AMPK (Shaw et al., 2005, Foretz et al., 2010). Metformin inhibits the complex 1 respiratory chain in mitochondria thus reducing the energy available for gluconeogenesis (Owen et al., 2000). Metformin may also have benefits in the treatment of tumour development as metformin fed PTEN -/- mice exhibit decreased tumorigenesis, and this action, like its regulation of gluconeogenesis, is also LKB1 dependent (Huang et al., 2008). Similarly there is evidence that DNA repair proteins may influence metformin action. Patients with ataxia-telangiectasia have a higher rate of insulin resistance and diabetes with rates of up to 59% (Schalch et al., 1970). The ataxia-telangiectasia mutated (ATM) protein can phosphorylate and activate AMPK in an LKB-1 independent manner (Fu et al., 2008, Sun et al., 2007), while ionising radiation induces AMPK phosphorylation in an ATM dependent manner (Sanli et al., 2010). This establishes a further link between metformin action and a reduction in neoplasia as the effects of metformin integrate pathways involved in DNA repair and nutrition, and this may explain the reduced cancer incidence in metformin users (Libby et al., 2009). Understanding how metformin reduces gluconeogenic gene expression

remains unknown, but could have major benefits for some of the most common diseases in our society.

1.12.2.2. Thiazolidindediones

The thiazolidinediones are PPAR-γ agonists. They have a multitude of effects in tissues that express PPAR-γ, mainly adipose tissue, pancreatic β-cells and vascular endothelium (Willson et al., 2001, Dubois et al., 2000). These drugs improve insulin sensitivity and lower both fasting and post prandial glucose, insulin and FFA (Nolan et al., 1994, Miyazaki et al., 2001). It is thought that the effects of thiazolidinediones are mainly due to an increase in the number of small adipocytes (Okuno et al., 1998). The increased number of adipocytes increases FFA uptake and thus reduces the triglyceride content of the liver. Furthermore, the larger number of adipocytes results in increased adiponectin production which itself has insulin sensitising effects on the liver (Maeda et al., 2001). This induction of adiponectin is proposed to lead to a reduction in levels of PEPCK and G6Pase mRNA independently of insulin action (Davies et al., 1999).

It is also of note that, like metformin, they also inhibit the mitochondrial respiratory chain, which is thought to underlie their ability to induce AMPK activity, although it remains to be seen if this has any role in the beneficial effects of these drugs (Brunmair et al., 2004).

1.12.3. Future Drugs to target hyperglycaemia and obesity

1.12.3.1. Sodium/Glucose Co-Transporter 2 (SGLT-2) Inhibitors

Sodium/glucose co-transporter 2 (SGLT-2) is a high capacity low affinity membrane transporter primarily present in the proximal segment of the proximal convoluted tubule of the nephron where the majority of glucose reuptake occurs (Kanai et al., 1994). In

contrast, SGLT-1 is expressed in the small intestine and distal proximal convoluted tubule, but has a greater affinity for galactose than SGLT-1 and is thought to be responsible for only minor amounts of renal glucose reuptake (Wright, 2001). SGLT-3 act as skeletal muscle glucose sensors (Wright et al., 2007). Inhibiting glucose re-uptake in the proximal convoluted tubule of the nephron with SGLT-2 inhibitors induces glycosuria and reduces hyperglycaemia. This is likely to have a significant clinical impact. Indeed, in high fat fed mice knocking out SGLT-2 prevented hyperglycaemia, hyperinsulinaemia and glucose intolerance (Jurczak et al., 2011). In diabetes, the re-uptake of glucose is increased by a higher expression of SGLT-2 channels (Rahmoune et al., 2005). Therefore a number of inhibitors of this channel have been developed. Phlorizin was the first compound indentified, but was unsuitable for clinical practice. Due to its non-specific nature it also inhibited SGLT-1 leading to osmotic diarrhoea (Perez Lopez et al., 2010). More recently, specific SGLT-2 inhibitors have been developed, with dapagliflozin the best characterised. In diabetes models inhibition of SGLT2 by dapagliflozin reduces plasma glucose but also improves insulin secretion, insulin sensitivity and hepatic glucose production (Macdonald et al., 2010, Han et al., 2008). In streptozotocin induced diabetes and Zucker diabetic rats, SGLT-2 inhibitors return hepatic glucose output to normal (Oku et al., 2000, Han et al., 2008). This may be due to a reduction in glucotoxicity and relief of inhibition of the insulin signalling pathway (Asano et al., 2004). However, it remains to be seen whether SGLT2 inhibitors are only of use in the presence of hyperglycaemia, which is absent in the first stages of the development of insulin resistance.

1.12.3.2. Human Fibroblast Growth Factor (FGF) 19

Human fibroblast growth factor 19 (FGF19) is produced in the small intestine in response to the uptake of bile acids. It has previously been shown to reduce levels of

both diet induced obesity and reverse diabetes in leptin deficient mice (Fu et al., 2004). These actions are independent of insulin action, most likely through the MAPK pathway, and selectively enhance glycogenesis without enhancing the lipolysis that leads to atherogenesis (Kir et al., 2011). Type 2 diabetes does not cause a deficiency of FGF19, and so the benefits of replacement are as yet unknown (Mraz et al., 2011).

1.13. Models of Insulin Resistance

1.13.1. Animal Models

A number of animal models of insulin resistance exist (Table1.2). These can be split into 3 main categories.

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1.13.1.1. Genetic models of obesity induced insulin resistance and type 2 diabetes

The evidence that insulin resistance in humans is closely related to obesity is very strong; therefore modelling insulin resistance normally involves generating obesity. There are a number of obese rodent models available. The earliest obese rodent model discovered was the Ob/Ob mouse. This occurred due to a spontaneous autosomal recessive mutation of the leptin gene (Enser, 1972, Friedman et al., 1991, Zhang et al., 1994). These mice develop obesity, hyperinsulinaemia and hyperglycaemia as early as four weeks of age (Dubuc, 1976). The db/db mouse arose due to another spontaneous autosomal recessive mutation, but this time of the leptin receptor (Chen et al., 1996). These mice develop obesity by 6 weeks of age, but do not show hyperinsulinaemia or glucose intolerance until 12 weeks (Dong et al., 2010, Winzell et al., 2010). The Zucker Diabetic Fatty (ZDF, Fa/Fa) rat was first identified in 1974, and arose due to the spontaneous mutation of the leptin receptor (Leonard et al., 2005). These rats develop

hyperphagia, obesity and insulin resistance (Leonard et al., 2005, Peterson et al., 1990, Finegood et al., 2001). They have a period of hyperinsulinaemic euglycaemia after which hyperglycaemia develops, usually by 7-12 weeks (Peterson et al., 1990, Leonard et al., 2005, Kuhlmann et al., 2003). These models have been most useful for the identification of leptin, and are now mostly used as a model of hyperphagia and obesity. This is due to the fact that the disruption of leptin has many actions unrelated to the phenotypes, and although there have been families identified with congenital leptin deficiency (Montague et al., 1997, Farooqi et al., 1999, Gibson et al., 2004), these remain vanishingly rare. The Goto-Kakizaki (GK) rat is a model of insulin resistance in the absence of obesity, which does exist in the human population but at a much lower prevalence than obesity related insulin resistance. Interestingly this rat develops dyslipidemia and fatty liver, however the mechanisms remain unclear hence it may not be representative of the human state of lean insulin resistance.

1.13.1.2. Genetically engineered mouse models

Transgenic and knockout technology has enabled the generation of mouse models lacking individual proteins or expressing mutant forms of specific proteins. Therefore it has been possible to remove individual insulin signalling proteins (see Table 1.1 and Chapter 1.6) and establish their role in insulin action and/or the development of insulin resistance and obesity. Once again, although many knockout mice (e.g. insulin receptor KO) readily develop insulin resistance or obesity or both, they often have much more severe phenotypes and are unlikely to be representative of insulin resistant states found in humans (or represent extreme forms of these diseases). The generation of hypomorphic or heterozygotic KOs where signalling pathways are reduced has been informative but also identified the existence of pathway redundancies. Again, the main issue with genetically deleting insulin signalling pathways as a model of the human

disease is that mutations in these pathways are extremely rare in humans with only one example, an AKT2 mutation resulting in severe insulin resistance, being identified in humans to date (George et al., 2004).

1.13.1.3. Diet induced obesity and insulin resistance

The obesogenic western diet rich in carbohydrates and saturated fat has similar effects on the metabolism of mice as it does on humans, particularly those kept relatively inactive in laboratory cages. The best example of this is the diet-induced obese (DIO) mouse which develops obesity when fed an *ad libitum* high fat diet, but remains lean when fed a low fat diet (Surwit et al., 1995). These mice develop gradually worsening insulin sensitivity associated with hyperinsulinaemia from 1 week, but the development of diabetes takes at least 3 months (Ahren and Pacini, 2002). However this model does mimic the development of insulin resistance and diabetes in man and is thus a useful way to determine efficacy of insulin sensitisers and anti-obesity drugs being developed for obese, insulin resistant populations.

1.13.2. Cell based models

Animal models are expensive and time consuming, therefore only the most promising therapeutic leads are taken to animal model studies. Therefore an important stage in pre-clinical drug development is finding cell based models of the disease to help identify leads with low toxicity and high efficacy and also provide information on mechanism of action. To date most insulin sensitising drugs are investigated in cell lines with 'normal' insulin sensitivity, efficacy being determined by improvements in insulin action in the absence of any defect in insulin signalling. This is not ideal however there is a complete lack of physiologically relevant insulin resistant cell models, with most being highly focussed and biased towards one or other of the many proposed mechanisms of obesity

induced diabetes (e.g. inflammatory markers, fatty acids and even hyperglycaemia). To induce insulin resistance cells are often cultured in comparatively extreme conditions of exposure to one or more agents related to obesity and insulin resistance. For example insulin resistance, as measured by complete loss of Tyr aminotransferase activity in response to insulin, can be induced in H-35 hepatoma cells by incubation for 4-6 hours in 50 nM insulin (Krett et al., 1983). It is also possible to induce insulin resistance with short term incubation of hepatoma cells with 30 mM glucose or high dose fatty acids (Nakamura et al., 2009, Zang et al., 2004). High dose fatty acids lead to a significant reduction in PKB phosphorylation (Nakamura et al., 2009). However, high glucose concentrations lead to a complete loss of insulin stimulated PKB and GSK-3 phosphorylation (Zang et al., 2004). In humans, there is not a complete loss of insulin action, but a shift in insulin sensitivity and as such these are poor models. Also, although these allow the rapid development of insulin resistance, they are not models of human insulin resistance which contains a milieu of factors, in physiological concentrations, all of which have effects on insulin sensitivity. Therefore an improved, more physiological and robust model of this disease is needed.

1.14. Aims and Objectives

- To optimise a cell based humanised model of insulin resistance,
- To develop luciferase reporter cell lines under the control of insulin responsive genes,
- Investigate which component (s) of serum from insulin resistant individuals is responsible for altering insulin sensitivity of cells,
- Characterise the intracellular signalling defects associated with the early development of insulin resistance in the model developed,
- Use the model to investigate the mechanism of action of metformin.

Model	Diabetes Mellitus	Phenotype	Reference
ob/ob Mice (leptin deficient)	Yes	Obesity, hyperinsulinaemia, hyperglycaemia, fatty liver, hypotension	(Dubuc, 1976) (Bigorgne et al., 2008)
db/db mice (leptin receptor mutant)	Yes	Obesity, dyslipidaemia, hyperglycaemia, hyperinsulinaemia, fatty liver	(Dong et al., 2010) (Ge et al., 2010)
Zucker Diabetic Fatty (ZDF) Fa/Fa rats (leptin receptor mutant)	No	Obesity, hyperinsulinaemia, dyslipidaemia, fatty liver	(Zucker and Zucker, 1961) (Chanussot et al., 1984)
Otsuka Long Evans Tokushima Fatty (OTLEF) rats CCK-1 receptor deficient	Yes	Obesity, hypertension, hyperlipidaemia, fatty liver	(Kawano et al., 1994) (Yagi et al., 1997)
Goto-Kakizaki (GK) rats	Yes	lean, Dyslipidaemia, fatty liver, β-Cell defect,	(Goto et al., 1976) (Portha et al., 2010)
IR Knockout	Yes	Neonatal death from ketoacidosis, hyperglycaemia, hepatic steatosis, postnatal growth retardation	(Joshi et al., 1996) (Accili et al., 1996)
Liver IR Knockout (LIRKO)	Yes	Hyperinsulinaemia, hyperglycaemia, dysregulation of hepatic gene expression, elevated hepatic glucose production,	(Michael et al., 2000) (Biddinger et al., 2008)
IRS-1 Knockout	No	Mild insulin resistance. growth retardation, decreased glucose uptake, hypertension, hyperlipidaemia, β-cell hyperplasia	(Abe et al., 1998) (Araki et al., 1994)
IRS2 Knockout	Yes	Hepatic and muscle insulin resistance, hyperinsulinaemia, no β-cell hyperplasia	(Kubota et al., 2000) (Withers et al., 1998)
IRS-1 and IRS3 dual knockout	Yes	Lipoatrophy, hyperglycaemia, hyperinsulinaemia, leptin deficiency	(Laustsen et al., 2002)
AKT2 knockout	Yes	Hyperglycaemia, hyperinsulinaemia, insulin resistance, β-cell compensation	(Cho et al., 2001a)
Diet Induced Obesity (DIO) mouse	Yes	Diet related obesity, insulin resistance, hyperglycaemia, hypoadiponectinaemia, leptin resistance, dyslipidaemia, islet dysfunction,	(Surwit et al., 1995)

Table 1.2 List of mouse models of insulin resistance

Chapter 2. Materials and Methods

2.1. Materials

2.1.1. Chemicals and Equipment

NU-PAGE Bis-Tris 4-12% precast gels, apparatus and NuPage buffers for SDS-PAGE, apparatus for Western blotting, molecular weight standards, Superscript™ III reverse transcriptase, TOPO TA cloning kit, PureLink™ Quick Plasmid Miniprep Kit and PureLink™ HiPure Plasmid Filter Maxiprep Kit were from Invitrogen (Groningen, The Netherlands). Mini ProteanTGX 4-15% precast gels, apparatus and molecular weight standards were from Bio-Rad® laboratories Inc (Hertfordshire UK). Dried skimmed milk (Marvel) was from Premier Beverages (Stafford, UK). T7 DNA Ligase, RQ1 RNase-Free DNase, RQ1 Stop Solution, Random Primers for cDNA synthesis, Luciferase assay kit and all DNA restriction enzymes were from Promega (Southampton U.K.). 75 cm^2 cell culture flasks and 6 well plates were from NUNC™ Thermo Fisher Scientific (Roskilde Denmark. Nitrocellulose membrane, Enhanced Chemiluminescence (ECL) reagent and Hyperfilm™ were from Amersham Biotech (Buckinghamshire, UK). Autoradiography cassettes with intensifying screen were from Kodak (Liverpool UK). CL-XPosure film and bovine serum albumin for protein estimation was from Pierce (Chester UK). UV Biophotometer was from Eppendorf (Hamburg, Germany). DMEM, penicillin/streptomycin and trypsin for tissue culture were from Gibco (Paisley, UK). Foetal bovine serum was from PAN Biotech (Aidenbach, Germany). NucleoSpinR Extract II PCR clean-up gel extraction Kit was from Machery Nagel (Düren, Germany). 0.22 μm cellulose filters were from Millipore Ltd (Norwich, UK). All DNA primers and probes, dexamethasone, phenformin, Metformin, Tri-reagent™, Phosphate buffered saline, 1-3-bromochloropropane, dimethylsulfoxide, Ponceau S (in acetic acid) solution and ethidium bromide solution were from Sigma-Aldrich (Dorset, UK).Taqman ® Universal PCR Master Mix, 96-well optical reaction plates and optical caps for reaction plates were from Applied

Biosystems (Foster City, CA, USA). Pipettes were from Grenier (Gloucester, UK). Cell scrapers were from Sarstedt (Nümbrecht, Germany). Actrapid insulin was from Novo Nordisk (Bagsværd, Denmark). 8- (4-Chlorophenylthio)-Adenosine 3′,5′-cyclic Monophosphate was from Calbiochem (San Diego, CA, USA).

All other chemicals were of the highest grade available and purchased from Sigma Aldrich (Dorset, UK) or Calbiochem (San Diego, CA, USA)

2.1.2. Small molecule Inhibitors and Activators

The structures of small molecule inhibitors and activators used in this thesis are shown in Figure 2.1

2.2. Methods

2.2.1. Preparation of buffers

Commonly used buffers are listed in Table 2.1.

Lysis buffer contains EDTA as a chelating agent for magnesium ions and therefore inactivates protein kinases, phosphates and proteases. The inclusion of EGTA is to chelate calcium ions thus inactivating calcium dependent kinases, phosphatases and proteases, whilst sodium fluoride and sodium pyrophosphate inhibit Ser/Thr phosphatases. Sodium Orthovanadate inhibits phosphoTyr phosphatases.

Buffer	
Cell Lysis Buffer	25 mM Tris/HCl (pH 7.4), 50 mM NaF, 0.1 M NaCl, 5mM EGTA. 1mM EDTA, 20 mM NaPyPi, 1% (v/v) Triton X 100, 0.1 (v/v) β-mercaptoethanol, 1mM Na_3VO_4, 0.27M sucrose and protease inhibitor cocktail tablet
TAE	40mM Tris–acetate and 20mM EDTA pH 7.4
PBS	137mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4
4 X Lauryl dodecyl sulphate (LDS) sample buffer (Invitrogen)	4.3M glycerol, 563mM Tris Base, 41.9mM Tris-HCl, 293mM LDS, 2mM EDTA, 7.5% (v/v) 1% Serva blue G250, 12.5% (v/v) 1% Phenol Red
MOPS Running Buffer (Invitrogen)	50mM MOPS ((3-N-morpholino)propanesulphonic acid), 50mM Tris, 3.46mM SDS, 1mM EDTA, pH7.7
Running Buffer (Bio-rad)	25 mM Tris, pH 8.3, 192 mM glycine, 0.1% (w/v SDS
Laemmli sample Buffer (Bio-Rad)	62.5 mM Tris-HCl, pH 6.8, 2& (w/v) SDS, 25% (v/v) glycerol, 0.01% (w/v) Bromophenol Blue
Transfer Buffer	25 mM Tris, 192 mM glycine, pH 8.3, 20% (v/v) methanol
TBST	20 mM Tris/HCL pH7.5, 150mM NaCl, 0.05% (v/v) Tween
2 x BES	50 mM N,N-Bis (2-hydroxyethyl)-2-aminoethanesulfonic acid sodium salt (BES), 280 mM NaCl, 1.5 mM $NaHPO_4$, pH 6.95
LB Medium	1% (w/v) Bacto-Tryptone, 0.5% (w/v) Bacto-Yeast Extract, 0.5% (w/v) NaCl pH 7.0

Table 2.1 List of commonly used buffers

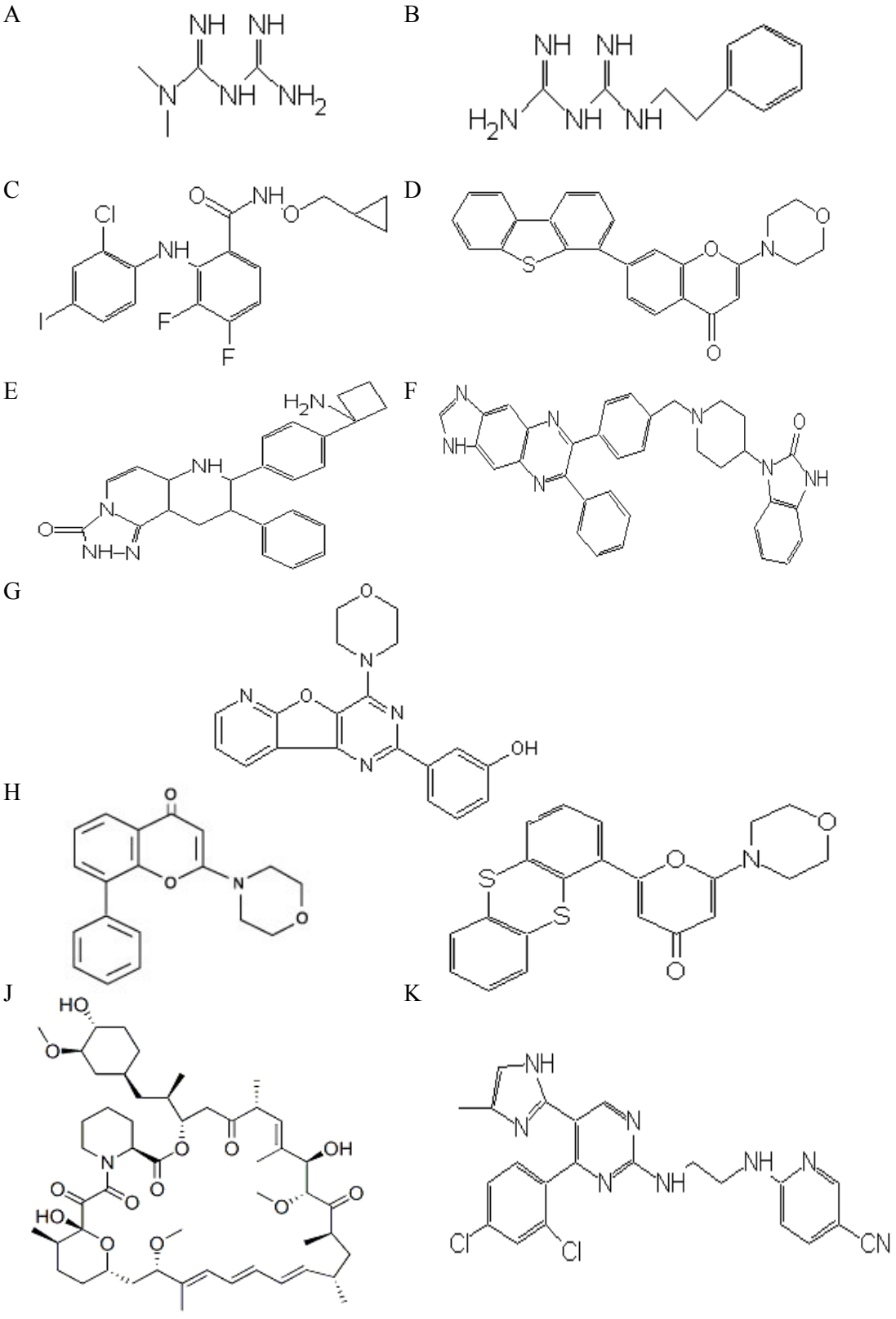


Figure 2.1 Structures of small molecules used in this thesis (A)Metformin, (B) Phenformin, (C)PD184352, (D)Nu7441, (E) MK2206, (F) Akti 1/2, (G) PI-103, (H) LY294002, (I) KU55993, (J)Rapamycin, (K) CT99021.

2.2.2. Cell Culture

Cell culture media were warmed to 37°C prior to use.

2.2.2.1. H4IIe cells

Rat hepatoma H4IIe cells were used as an insulin responsive cell line. The cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) containing 1g/L glucose, 580 mg/L L-glutamine and 110 mg/L sodium pyruvate (Gibco cat no:31885-023). This was supplemented with 5% (v/v) foetal calf serum (FCS) (PAN cat no 3302-P291205) and 1% (v/v) penicillin/streptomycin solution (Gibco cat no: 15140-122). This is referred to as complete media. Cells were cultured in 75cm^2 flasks at 37 °C with 5% CO_2 in a water saturated incubator. The cells were passaged twice weekly after reaching 80 -90% confluence. This was performed by aspiration of the culture medium followed by washing with 5ml of sterile PBS. The PBS was aspirated and 1ml of 0.05% Trypsin/EDTA (Gibco cat no: 25300-054) added. The cells were incubated for 3-5 minutes until they were detached. The trypsin was neutralised by the addition of 4 ml of fresh complete media and the resulting suspension thoroughly mixed. 1 ml of the suspension was transferred to a sterile 75 cm^2 flask and made up to a final volume of 10 ml with complete media. Alternatively, cells were counted using a cytometer and used to seed 6 well plates at a concentration of 1 x 10^6 cells per well for gene expression studies, or to plate at 50% confluence in 6 or 10 cm dishes.

2.2.2.2. Reporter cell lines (LLRP7, CSHP12, LLHG4, CSHI4)

The generation of these cells is described under Results. They were maintained in Dulbecco's Modified Eagle's medium (DMEM) containing 1g/L glucose, 580 mg/L L-glutamine and 110 mg/L sodium pyruvate (Gibco cat no:31885-023), containing G418 (500µg/ ml). Cells were cultured in 75cm^2 flasks at 37 °C with 5% CO_2 in a water

saturated incubator. Media was replaced once weekly and the cells passaged once weekly. Prior to their use in any experiments the cells were passaged into complete media without G418 and were cultured for 2 weeks as per H4IIe (Chapter 2.2.2.1).

2.2.2.3. Freezing cells for storage

Aliquots of all cell lines were frozen for long term storage as follows. The culture medium from a confluent 75cm^2 flask was removed by aspiration followed by washing with 5ml of sterile PBS. The PBS was aspirated and 1ml of 0.05% Trypsin/EDTA added. The cells were incubated for 3-5 minutes at 37 °C with 5% CO_2 in a water saturated incubator until they were detached. The trypsin was neutralised by the addition of 4 ml of fresh complete media and the resulting suspension thoroughly mixed. The cells were transferred to a clean 50 ml centrifuge tube and centrifuged at 3,500 rpm for 4 minutes. The media was aspirated and washed once with 5 ml PBS. The cells were centrifuged at 3,500 rpm for 4 minutes, the PBS aspirated and the cells suspended in 3 ml complete medium containing 10% (v/v) DMSO and thoroughly mixed. 1 ml of cell suspension was transferred to a 1ml CryoTube™, placed in a Cryo 1°C Freezing container (Nalgene) and transferred to a freezer maintained at -80°C. After 48 hours, vials were transferred to liquid nitrogen for long term storage.

2.2.2.4. Stimulation and harvesting of cells for RNA extraction

Prior to treatment with hormones, compounds or inhibitors, H4IIe cells were counted with a haemocytometer and plated at a concentration of 1 x 10^6 cells per well of a six well plate. Cells were left to attach overnight. In all cases cells were washed with sterile PBS and incubated in serum free media for 3 hours (fasted) prior to treatment. Cells were then treated for 3 hours with serum free media (Serum Free), 500 nM Dexamethasone and 0.1 mM 8 CPT-cAMP (Stimulated) with or without the addition of

insulin (as indicated in Figure legends). If inhibitors were used, cells were incubated with these for 30 minutes prior to hormone treatment.

2.2.3. RNA extraction from cells, cDNA preparation and analysis by Taqman

Total cellular RNA was extracted using guanidinium isothiocyanate (TRI Reagent®), a chaotropic agent that denatures cell membranes, DNA and proteins whilst keeping the integrity of RNA intact. This is then mixed with chloroform, a trihalomethane that, when added to cell lysates containing guanidinium isothiocyanate separates the RNA (aqueous phase) from the protein (layers between aqueous and organic phases) and DNA (organic phase). Isopropanol is added to the isolated aqueous phase to precipitate the RNA.

2.2.3.1. RNA extraction

Following the treatment of H4IIe cells under appropriate conditions, media was aspirated and cells washed once in 1ml of sterile PBS per well of a 6 well plate. PBS was aspirated and 400 µl of TRI-reagent added to each well. Each well was scraped with a cell scraper to disrupt the cell membranes and the lysate transferred to a 1.5ml microcentrifuge tube and kept on ice. 40 µl of 1-bromo-3-chloropropane was added to each tube, mixed well by inversion and left at room temperature for 5 minutes. The tubes were then centrifuged for 15 minutes at 13,000 rpm at 4ºC. The clear aqueous layer (upper) was transferred to a clean microcentrifuge tube, 200 µl of isopropanol added and mixed well by inversion. This was left to stand at room temperature for 5 minutes to allow precipitation of RNA then centrifuged at 13,000 rpm for 10 minutes at 4 ºC to create a pellet. The supernatant was removed, 1ml of 75% ethanol added to gently wash the pellet followed by centrifugation at 13,000 rpm for 5 minutes at 4 ºC. The supernatant was removed and the pellet allowed to dry at 50 ºC for 5 minutes. The

resultant pellet was suspended in 30 µl of nuclease free water. The resuspended RNA was transferred to a 96 well PCR plate. 3µl each of DNase buffer and RQ1 DNase were added to each well and incubated at 37 ºC for 30 minutes in a thermocycler to degrade any contaminating DNA. RQ1 DNAse requires the presence of magnesium and calcium ions contained in the reaction buffer. Following this, 3 µl of DNase stop solution which chelates the magnesium and calcium ions and terminates the reaction was added to each well and incubated at 65 ºC for 10 minutes in a thermocycler to allow heat denaturation of the enzyme. The purified RNA was stored at -80°C until required.

2.2.3.2. Measurement of RNA and DNA Concentration

Nucleic acids absorb light at a wavelength of 260 nm (A260) and proteins at a wavelength of 280 nm (A280). The concentration of RNA was determined using a spectrophotometer set at a wavelength of 260 nm with one absorbance unit equal to 40µg/ ml. 5 µl of the DNase treated RNA sample was added to 400 µl of nuclease free water in a Uvette® (Eppendorf) and the RNA concentration calculated. The ratio of A260/280 gave a measure of the purity of RNA. Pure RNA has an A260/280 of 2. Only RNA with a ratio of greater than or equal to 1.6 was used for further assays.

DNA was diluted 1:80 into RNAse free water in disposable UVette cuvettes (Eppendorf) and the absorbance measured at 260 and 280 nm using a UV Biophotometer (Eppendorf). An absorbance of 1 is equivalent to 50 ng/ ml of double stranded DNA. The ratio of A260/A280 indicates the purity of the sample and pure DNA would have an A260/A280 of 1.8.

2.2.4. cDNA synthesis for Real-Time PCR

Reverse transcriptase polymerase chain reaction was performed using the Superscript III reverse transcriptase system from Invitrogen. This method uses an engineered mutant of the Moloney Murine Leukaemia Virus (M-MLV) reverse transcriptase enzyme with increased thermal stability allowing synthesis of first strand cDNA. All reactions were performed in a thermocycler. 1µg of total RNA was aliquoted into a well of a clean 96 well PCR plate and the total volume made up to 11 µl with nuclease free water. In a clean microcentrifuge tube a mix containing a 0.25:0.75:1 ratio of random hexamers, nuclease free water and 10mM dNTPs was prepared and 2 µl of this mixture added to each well to prime the RNA for cDNA synthesis. The samples were mixed by vortex and briefly centrifuged before incubation for 5 minutes at 65 ºC. In a clean centrifuge tube a mix containing a 4:1:1:1 ratio of 5x first strand buffer, 0.1 M DTT, RNAse inhibitor and Superscript III was prepared and 7 µl of this mix was added to each well. The solutions were mixed by vortex followed by brief centrifugation and incubated at 25 ºC for 5 minutes then 50 ºC for 50 minutes and finally 70ºC for 15 minutes. 180 µl of nuclease free water was added to each well (10x dilution) and cDNA samples were stored at -20 ºC until required for cDNA amplifications.

2.2.5. Polymerase Chain Reaction (PCR)

Polymerase Chain reaction is a semiquantative technique used to exponentially amplify DNA sequences. KOD Hot Start DNA Polymerase (Merck) and specific oligonucleotide primers (Sigma-Genosys) were used for all cloning reactions. KOD Hot Start Polymerase utilizes a premix of a DNA polymerase (KOD) with two monoclonal antibodies that inhibit the DNA polymerase and 3’ to 5’ exonuclease activity at ambient temperature. The DNA polymerase exhibits high fidelity and proof reading ability and comes from the *Thermococcus kodakaraensis*. PCR reactions are shown in Table 2.2.

2.2.5.1. Reverse Transcriptase polymerase chain reaction (RT-PCR)

RT-PCR differs from conventional PCR insomuch as the initial template is mRNA. This two step PCR strategy includes an initial generation of a single cDNA molecule from every mRNA molecule in the sample of interest (Chapter 2.2.4). The second step is a standard PCR reaction or more quantitative Real-Time PCR.

2.2.5.2. Real-Time PCR (Taqman^{TM})

Taqman is a method used to quantify the absolute levels of a specific DNA (cDNA generated from mRNA when applied to measurement of gene expression) sequence in a sample. The template cDNA is primed and amplified in consecutive rounds of annealing, extension by polymerase and denaturing of the double stranded structure. In contrast to standard PCR, Taqman is designed to quantify the accumulation of the amplicon after every amplification cycle rather than at a single point.

This process requires 3 specific oligonucleotides to bind to the target cDNA sequence. Two primers bind to the end of the sequence to be amplified and a labelled probe hybridizes to the target sequence between the primers. The probe is a complimentary oligonucleotide to the target DNA sequence covalently bonded to a fluorophore at the 5' end and a quencher at the 3' end. All reactions described here use a 5' 6-carboxyfluorescein (FAM) modification and a 3' 6-carboxy-tetramethylrhodamine (TAMRA) modification. As the fluorophore and quencher are in close proximity, any fluorescent activity is quenched.

During amplification the probe binds to the specific DNA region within the amplicon. As the primers are extended by the polymerase and the complementary strand is produced, the intrinsic 5’ to 3’ exonuclease activity of Taq polymerase breaks down the

Primer Name	Primer Sequence (5' to 3')	Melting Temp (◦C)	Annealing Temp (◦C)	No of Cycles
Human G6PAse Forward (hG6Ps5prSalI)	GTCGACCCTTTGAGAATCCACGGTGTC	75.3	68	35
Human G6PAse Reverse (hG6Ps3prHind)	AAGCTTAGGTGCCAAGGAAATGAGG	69.7		
Human IGFBP-1 Forward (hBP15prSalI)	GTCGACGTTCCCAGAAATGGTGTGTG	77.0	68	35
Human IGFBP-1 Reverse (hBP13prHind)	AAGCTTAAACTCTGGGCAAGTGATGC	69.7		
Human PEPCK Forward (humPCKf)	ATTCCTTTCTCGACCCTCGTC	65.5	62	30
Human PEPCK Reverse (humPCKr)	ATCTCGAAGGGAGATCCACAG	64.4		
Rat PEPCK Forward (PCRPCKfor)	GTTACGGTACCGTACGAGCGCTGAACATCACAC	78.2	68	35
Rat PEPCK Reverse (PCRPCKrev)	GCTTACGCTCGAGCTCCTTTTGGAAGGTCTGGA	79.9		

Table 2.2 PCR cycles used in this thesis
The pairs of primers were used in the presence of KOD hot start polymerase under the following conditions

1 - 95°C for 2 minutes
2 - 95 °C for 20 seconds
3 - Annealing temp for 10 seconds
4 - 70°C for 1 minute

Steps 2-4 were repeated for the number of cycles as shown

probe. This releases the probe from the proximity of the quencher, thus allowing the fluorophore to fluoresce. Therefore, the fluorescence increases with amplification and is directly proportional to the number of new amplicons generated at each cycle. Therefore the greater the number of cDNA molecules of the probe target sequence present at the start of the reaction the less cycles are required to reach predefined fluorescence levels.

Reporter fluorescence values are detected and measured at the end of every cycle of amplification. The fluorescence is proportional to the quantity of amplified product and is recorded as relative fluorescence against cycle number. The first 15 cycles are used to generate average background fluorescence. This is generated by variations in fluorescence resulting from the background media and prior to significant amplification of the target sequence. During the PCR process, a point is reached at which the fluorescence signal is significantly higher than the calculated background value. This is known as the threshold cycle and is used as a benchmark for further quantification. The threshold is reached during the period of most rapid change in the reaction and avoids the limiting factors which may occur in the plateau phase, i.e. self annealing of DNA products and competition for primer binding. The quantification is based around the concept that the greater the amount of template at the start of the reaction, the fewer cycles will be required for the fluorescence to reach threshold and thus the threshold cycle is inversely proportional to the starting copy number of template DNA. When fluorescence of the reporter dye reaches this level, it is this Cycle Threshold (Ct) value which can be converted to the mRNA copy number using the following algorithm

$$\text{Copy Number} = 2^{(40-Ct)}$$

The greater the Ct value, the greater the number of cycles it takes for detection of reporter fluorescence, and the fewer copies of mRNA are present.

250 ng of template cDNA was added to each Taqman reaction and mixed with 5µl 2x TaqMan® Universal PCR master mix (Applied Biosystems cat no: 4324018, containing reaction buffer, dNTPs and AmpliTaq Gold® DNA Polymerase), 0.5µl of 5µM forward primer (final concentration 200 nM), 0.5µl of 5µM reverse primer (final concentration 200nM), 0.25µl of 5µM probe (final concentration 100 nM) and 3.75µl of nuclease free water. Therefore, the total reaction volume for each sample was 12.5 µl. All samples were analysed in triplicate. Instruments used for Real-Time PCR use fluorescence to quantify PCR products as they accumulate. The ABI Prism 7700 (Perkin-Elmer-Applied Biosystems) detects fluorescence in the 500-660 nm range. Reactions are performed in a 96 well plate and fluorescence is induced by a 10 mW Argon Ion Laser. Fluorescent emission is detected using a 64x512 pixel CCD detector. The PCR reaction was carried out as follows: 50 ºC for 2 minutes, 95 ºC for 10 minutes and forty cycles of 95 ºC for 15 secs followed by 60 ºC for 1 min.

2.2.5.3. Primer/Probe design

Taqman primers are designed to have a melting temperature (Tm) between 58 and 60 ºC, a G/C content of 20-80% and a length of 90-40 nucleotides. Ideally, the difference in Tm between the primers should be less than 2 ºC and the last five nucleotides at the 3' end should contain a maximum of 2 G/C nucleotides. Primers were designed to flank exon-intron boundaries and amplify a region of approximately 150 bp of the cDNA. Probes were designed to have a Tm 10 ºC higher than the primers. The probes also had a G/C content of 20-80%, guanine nucleotides were excluded from the 5'end and there were no more than 3 contiguous guanine nucleotides present in the sequence. All primers contained more cytosine than guanine nucleotides. All probes had a 5' FAM dye and 3' TAMRA modification. Primers used for Taqman analysis are shown in Table 2.3.

2.2.6. Agarose Gel electrophoresis

Agarose gel electrophoresis allows the separation of DNA species, and for linear species the separation is mainly on the basis of molecular weight. Uses include visualization of the products of PCR reactions (allows a check of the success and purity of the reaction), and analysis of restriction digests. Agarose gels were 1% (w/v) unless otherwise stated. They were made with molecular biology grade agarose in 1X TAE buffer (Table 2.1). Ethidium Bromide (5µl of 10mg/ ml per 100 ml gel) was added to the molten agarose gel prior to pouring and cooling. Ethidium Bromide intercalates with DNA and fluoresces under UV light, this fluorescence increasing after intercalation and concentration. Gels were run for 60-80 minutes at a constant 55V and all gels included a lane of a standard DNA size ladder. DNA was visualized by a UV light in a transilluminator.

2.2.7. Agarose Gel Purification

After electrophoresis, DNA was extracted from the gel using the NucleoSpin® Extract II PCR clean-up gel extraction Kit (Machery Nagel). This method allows the binding of DNA to a silica membrane in the presence of chaotropic salt. The membrane is washed with an ethanolic wash buffer before pure DNA can be eluted in nucleic acid free water. The required DNA band was visualized under ultraviolet (UV) light and excised from the agarose gel using a clean scalpel and placed in a clean microcentrifuge tube. The weight of the slice was determined and 200µL of buffer NT was added for each 100 mg of gel. The mixture was incubated for 10 minutes at 50°C; with vortexing every 2-3 minutes until the sample was completely dissolved. The sample was loaded into a clean NucleoSpin® Extract II column in a collection tube and centrifuged at 13,000 rpm for 1 minute. The flowthrough was discarded and the column returned to the collection tube.

Rat Gene of Interest		Sequence (5'→3')
PEPCK	F	AAGGGCAAGGTCATCATGCA
	R	TGCCGAAGTTGTAGCCAAAGA
	Pr	FAM-ACCCCTTCGCTACTGCGGCCC-TAM
G6Pase	F	CTCCAGCATGTACCGCAAGA
	R	GGCTTCAGCGAGTCAAAGAGA
	Pr	FAM-AGTCGCTCCCATTCCGTTTGGCC-TAMRA
IGFBP-1	F	GCTGGATAGCTTCCACCTCATG
	R	TCCATTTCTTGAGGTCAGTCAGTGATCTC
	P	CCCCATCCCGTGAGGACCAGC
Actin	F	TCTGTGTGGATTGGTGGCTCTA
	R	CTGCTTGCTGATCCACATCTG
	Pr	FAM-CCTGGCCTCAATGTCCACCTTCCA-TAMRA

Table 2.3 Primers and probes used for Taqman analysis (F=)forward, R=reverse, Pr= probe

700 µl buffer NT3 was added to the column and centrifuged for 1 minute at 13,000 rpm. The flow through was discarded and the column returned to the collection tube. The column was centrifuged for 2 minutes at 13,000 rpm to dry the silica membrane. The column was placed in a clean 1.5 ml microcentrifuge tube and 30 µl of nucleic acid free water was to the membrane. This was incubated at room temperature for 1 minute before centrifuging for 1 minute at 13,000 rpm. The elute was frozen at -20°C until required.

2.2.8. Purification of plasmid DNA

Plasmids were extracted and purified using PureLink™ Quick Plasmid Miniprep Kit (small preparations) and PureLink™ HiPure Plasmid Filter Maxiprep Kit (large preparations). All procedures were performed according to the manufacturer's protocol using buffers supplied with kits.

For small plasmid preparations, 5 ml bacterial cultures were pelleted for 10 min in a Sigma 3K10 bench top centrifuge. The supernatant was removed; bacterial pellets were resuspended in 250 uL Buffer (R3) and transferred to 1.5 ml microcentrifuge tubes. The cells were lysed by the addition of Buffer L7 and lysis was terminated by the addition of Buffer N4. Samples were spun for 10 min at 13,000 rpm in a benchtop centrifuge. The supernatant was transferred to a spin column. The DNA was bound to the column by centrifugation for 1 min at 13,000 rpm and washed by adding 0.75 ml Buffer W10. The column was spun at 13,000 rpm for 1 minute, the flowthrough discarded and the column re-spun for 2 minutes to ensure all the ethanol was removed. DNA was eluted from the column in 50 µl nuclease free water, quantified and stored at -20°C

For large scale plasmid preparations (Maxiprep), 200ml bacterial cultures were grown and harvested by centrifugation at 4000 rpm for 10 minutes. The pellets were resuspended in 10ml Resuspension Buffer R3 containing RNase A and the suspension mixed until homogeneous. The bacteria were lysed by adding 10 ml Lysis Buffer L7 and allowing to stand at room temperature for 5 minutes. Lysis was terminated by the addition of 10 ml of Precipitation Buffer N3 and mixed until homogeneous. HiPure Filter Nidi Columns were prepared by adding 30 ml Equilibration Buffer EQ1 to the filtration cartridge and this was drained by gravity. The DNA was added to the column and drained by gravity. The filtration cartridge was discarded and the column containing bound DNA was washed using Wash Buffer W8 and drained by gravity. A 50 ml centrifuge tube was placed under the column and the DNA eluted by adding 15 ml Elution Buffer E4 to the column and allowing to drain by gravity flow. To precipitate the DNA, the eluate was incubated at room temperature with 10.5 ml 100% isopropanol for 2 minutes and collected by centrifugation at 13,000g for 30 minutes at 4°C. The supernatant was discarded and the pellets air-dried at room temperature for 30 minutes prior to resuspension in 500 µl RNAse free water. The DNA was quantified and then frozen at -20°C until required.

2.2.9. Restriction enzyme digests of DNA

Restriction Enzymes are endonucleases that cut single or double stranded DNA at specific nucleotide sequences. They are most commonly found in, and purified from, lower organisms. These enzymes have revolutionized molecular biology, allowing site specific cutting of DNA sequences, prior to ligation into alternative DNA sequences, often termed sub-cloning. In this way a specified piece of DNA can be moved from one genetic location to another, or multiple sequences ligated in series, by simple restriction digest, purification of fragments and ligation into new target sequence. A standard

restriction enzyme reaction combined 1 µg plasmid or PCR product, 1U of the restriction endonuclease, 1ul of 10X appropriate digestion buffer and 1 µl BSA (10 mg/ ml) to a final volume of 10 µl. Reactions were carried out at the appropriate temperature for maximal enzyme activity.

2.2.10. DNA Ligations

After DNA has been digested, the products were separated by agarose gel electrophoresis and purified as previously described (Chapter 2.2.6 and 2.2.7). For ligation reactions, 1µl purified linearised target vector (either with two distinct restriction ends to match the proposed insert, or dephosphorylated to prevent self ligation) was mixed with 1-7 µl of the insert to be ligated with 1 U of T4 DNA ligase and 1 µl 10X ligation buffer to a total volume of 10 µl. The reaction was carried out at 4°C for 16 hours. The ligation reaction was then transformed into chemically competent bacteria (Chapter 2.2.11), and antibiotic selection of bacterial clones containing the ligation product performed dependent on the resistance marker encoded by the target vector. Control ligation reactions containing no insert were also performed to ensure no self ligation of vector occurred.

2.2.11. Bacterial Transformation

For each transformation, 50 µl of chemically competent XL-1 Blue *E.Coli* were added to the plasmid. The cells were incubated on ice for 30 minutes prior to heat shocking at 42 °C for 90 seconds to aid DNA uptake and then immediately returned to ice for 2 minutes. 500 µl of LB medium was added to the mixture and incubated at 37 °C for 1 hour. The bacteria were spread onto agar plates containing 50 µg/ ml ampicillin or 50 µg/ ml kanamycin and allowed to air dry before overnight incubation at 37°C. The DNA vector contains an antibiotic resistance marker which will only be expressed if the

vector is circular. Therefore, only bacteria containing this will grow, whilst those containing the linear, or no, vector will not.

2.2.12. TOPO TA® cloning ligations

The TOPO TA® system (Invitrogen) uses a highly efficient one step cloning process for the direct insertion and propagation of PCR products into a plasmid vector which confers dual resistance to ampicillin and kanamycin. The linearised pCR 2.1® TOPO plasmid has a single 3' thymidine overhang and the DNA is covalently bound to Topoisomerase I at both the 5' and 3' thymidine phosphates of the linearised vector. The Topoisomerase, which originates from *Vaccinia* virus, cleaves the phosphodiester backbone of the vector. When the PCR product is mixed with the vector the topoisomerase allows the rapid ligation of the product into the vector followed by release of the enzyme rendering it inactive.

PCR products to be ligated into pCR 2.1® TOPO were purified by agarose gel electrophoresis prior to purification as previously described (Chapter 2.2.6 and 2.2.7). Reactions were performed by adding 4 µl of the purified PCR product to 1 µl salt solution (1.2M NaCl, 0.06M $MgCl_2$) and 1 µl of pCR 2.1® TOPO, prior to incubation at room temperature for 15 minutes and chilling on ice. The TOPO ligation reaction was then transformed into TOP10 One Shot® competent cells (Invitrogen). The contents of the ligation were mixed with the competent cells and incubated on ice for 30 minutes. Following this, the mixture was heat shocked at 42°C for 30 seconds to aid DNA uptake and immediately placed back on ice and incubated for 2 minutes. 250 µl S.O.C. media (2% (w/v) Tryptone, 0.5% (w/v) Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM Glucose) was added to the mixture and incubated at 37°C for 1 hour. The transformed bacteria were plated on agar plates containing 50 µg/

ml kanamycin, 10 mM IPTG and 100 µl 2% X-Gal. The plates were air dried and incubated overnight at 37°C. Vectors into which the insert has successfully ligated grow as white colonies on agar plates containing IPTG and X-gal because the insert disrupts a β-galactosidase expression cassette in TOPO. Therefore white colonies are selected and blue colonies (no insert- self ligation of TOPO) discarded. At least 8 white colonies from each cloning reaction were routinely selected and grown in Luria Broth containing 50 µg/ ml ampicillin before purification of the DNA as previously described (Chapter 2.2.8).

2.2.13. DNA sequencing

All Sequencing of PCR products and plasmids were carried out by Genetic Core Services Unit, Division of Medical Sciences, Ninewells Hospital, University of Dundee.

2.2.14. Protein Extraction from cells

Media was removed by aspiration and the cells were washed twice in ice cold PBS and aspirated to dryness. Cell lysis was performed by the addition of 100 µl (6 well plate), 200 µl (6 cm dish) or 500 µl (10 cm dish) of ice cold lysis buffer (Table 2.1). Dishes were placed on ice and the cells disrupted by scraping into lysis buffer and the lysates transferred to microcentrifuge tubes. The samples were snap frozen in liquid nitrogen and thawed on ice before cellular debris was removed by centrifugation at 13,000 rpm for 15 minutes at 4°C. The supernatant was transferred to a clean microcentrifuge tube, snap frozen and stored at -20 °C.

2.2.14.1. Measurement of Protein Concentration using the Bradford Method.

Bio-Rad Protein Assay (Bio-Rad) was used to determine the concentration of protein in cell lysates. This is a modified colorimetric assay first developed by Bradford (Bradford, 1976). The reagent contains Coomassie brilliant blue G-250, a dye which changes colour when bound to protein. This occurs due to binding of basic and aromatic amino acid residues to the dye producing a maximum absorption of 595 nm. The quantity of absorption is directly proportional to the bound dye and thus in theory also the amount of protein in the sample. Importantly the reading is only directly related to protein concentration between a specific range of protein concentrations, in this assay around 200μg/ ml to 1400 μg/ ml. The quantity of protein in an unknown sample can be calculated by comparing the level of absorption in a sample at 595nm with a standard curve (which also confirms the range of linearity of each assay).

A standard curve was generated by determining the A595 readings of serial dilutions of the inert protein BSA. Dilutions containing 0, 0.125, 0.25, 0.5, 1 and 2 μg/μl in lysis buffer were made and 5 μl of each was transferred to disposable plastic cuvettes. The Protein assay reagent was diluted 1:5 in distilled water; 1ml was added to each cuvette and mixed thoroughly by vortexing. The cuvettes were incubated at room temperature shielded from the light for 10 minutes prior to the absorbance being read at 595nm. A standard Bradford curve is shown in Figure 2.2.

2.2.15. Immunoprecipitation of proteins from cell extracts

Protein G from bacteria binds non-covalently to the Fc region of IgG antibodies. The protein can be covalently linked to Sepharose beads and then used to immunoprecipitate specific proteins from cell lysates by centrifugation. Cell extract (at 1 μg/μL, between 100 and 1000 μg total as indicated in Figure legends) was incubated with 1 μg primary

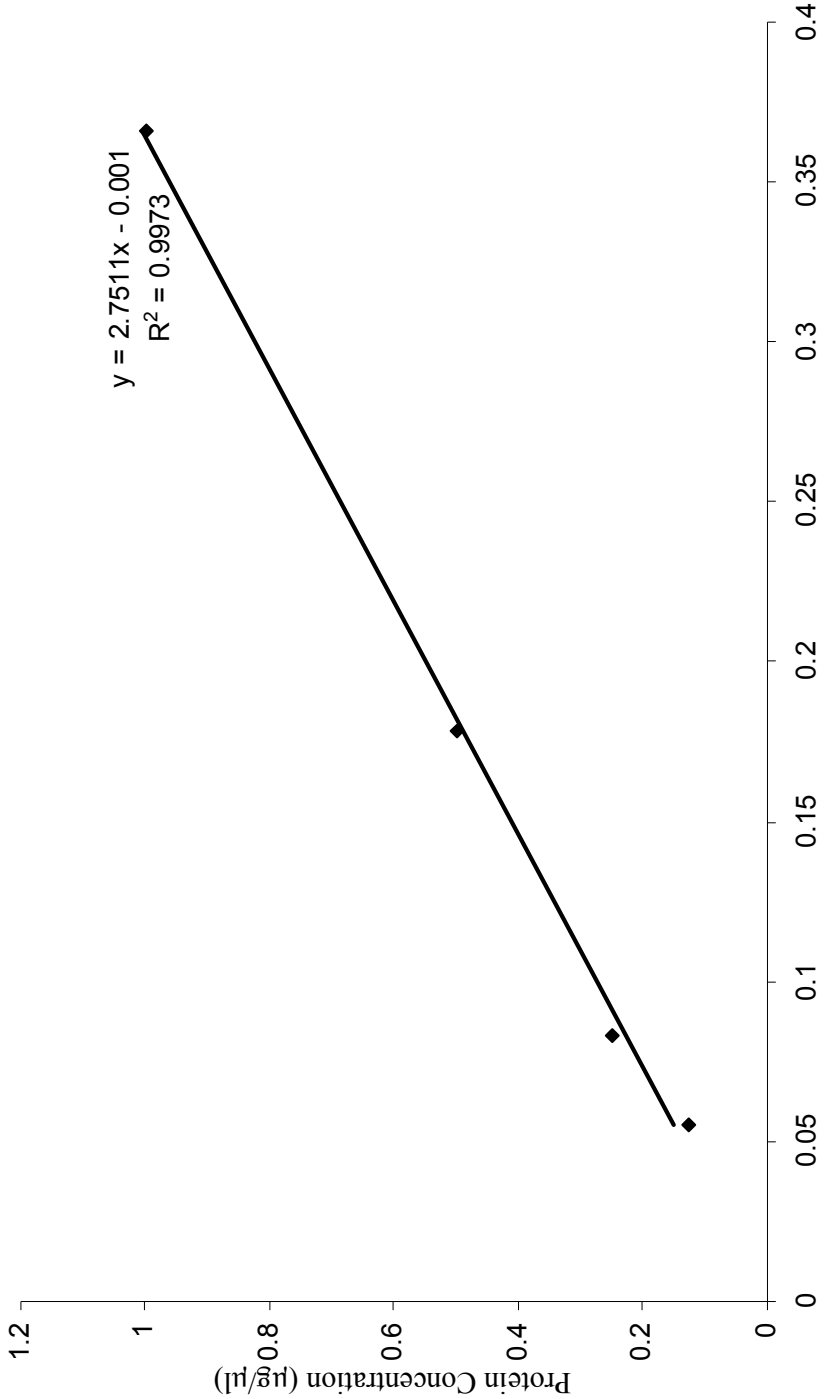


Figure 2.2 Example of a Bradford assay standard curve

antibody overnight at 4°C on a shaking platform. The following morning the Protein G sepharose beads were washed into lysis buffer. Specifically, 10 μL (per IP) of a 50:50 bead slurry was washed twice in 1X lysis buffer lacking sodium orthovanadate or β-mercaptoethanol or protease inhibitors. Each step was followed by centrifugation at 2000 rpm for 30 seconds at 4°C followed by aspiration of supernatant. The beads were then diluted to a 50:50 slurry with lysis buffer. 10 μL of the bead slurry was added to each sample and incubated at 4°C for 3 hours in a shaking platform. The immunocomplexes were pelleted by centrifugation at 2000 rpm for 0.5 min, and the supernatant transferred to clean microcentrifuge tubes. The beads were washed once in 1 ml of lysis buffer containing 0.5M NaCl, followed by 2 further washes in lysis buffer alone. Bound proteins were released from the beads by denaturation at 95°C in SDS PAGE loading buffer for 5 minutes followed by visualization by SDS-PAGE and Western blotting (Chapters 2.2.17 and 2.2.18).

2.2.16. Cell lysis for PIP3 measurement

H4IIe cells were plated at 60-80% confluence. Cells were fasted for 16 hours prior to 10 minutes treatment in the presence or absence of insulin. Media was removed by aspiration and 1 ml of 0.5M trichloroacetic acid added. The cells were disrupted by scraping with a cell scraper and a further 0.5 ml of ice cold 0.5M trichloroacetic acid added. This was then transferred to a clean microcentrifuge tube and centrifuged at 13,000 rpm for 15 minutes at 4°C. The acid was carefully removed and the pellet snap-frozen in liquid nitrogen and stored on dry ice. The pellets were delivered to Dr Alexander Gray at the College of Life Science, Dundee to analyse PIP3 levels using a previously described method (Gray et al., 2003).

2.2.17. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is a technique that separates proteins according to their electrophoretic mobility as determined exclusively by their molecular weight. An anionic surfactant, sodium dodecyl sulphate (SDS), denatures proteins by disrupting the secondary structure and imparting a negative charge. This means that during gel electrophoresis the denatured proteins separate by their molecular weight rather than being affected by the mass:charge ratio. Larger proteins move more slowly through the pores of the acrylamide matrix than smaller ones. Prior to electrophoresis, cell lysates were denatured in 4X SDS sample buffer containing 10 mM DTT by heating at 95 ˚C for 10 minutes. Prestained See Blue Plus2 markers (Invitrogen) or Precision Plus Protein Prestained Standards (Bio-Rad) were used on every gel as molecular weight standards.

Protein electrophoresis was performed using either NuPAGE® Novex 4-12% gradient Bis-Tris (Invitrogen) or Mini-PROTEAN TGX 4-15% (BioRad) pre-cast gels. Invitrogen gels were run in Xcell SureLock TM Mini-Cell tanks containing 1 X NuPAGE® MOPS running buffer (Invitrogen) and NuPAGE antioxidant® (Invitrogen). Mini-Protean gels were run in the mini-PROTEAN tetra system tank containing 1 X Tris/glycine/SDS running buffer (BioRad). Gel tanks were run at 110V for the first 10 minutes and then at 180 V for a further 70 minutes. When visualizing proteins of >170 kDa a total running time of 2 hours was used.

2.2.18. Western Blotting

After separation of proteins by SDS-PAGE, they were transferred to nitrocellulose membranes using the Trans Blot cell system (Bio-Rad). Blotting pads, pre-cut 3mm filter paper and Hybond ECL nitrocellulose membranes (Amersham) were rinsed in distilled water and then soaked in 1X transfer buffer (Table 2.1) containing 20% (v/v)

methanol. The SDS-PAGE apparatus was carefully disassembled, the gels removed from their cartridges and also soaked in transfer buffer. The Western blot sandwich was assembled and loaded into the Trans Bolt tank and the tank filled with transfer buffer as per manufacturer's instructions. Gels were transferred at 30V for 2 hours.

The efficiency of protein transfer was assessed by soaking the membranes in 0.1% (w/v) Ponceau S (Sigma). The membranes were then washed in 1X TBST (Table 2.1) to destain and blocked in TBST containing 5% (w/v) fat free milk for 1 hour. Blots were then incubated overnight at 4°C with primary antibody (as indicated in Table 2.3). The following morning blots were washed 3 times with 1X TBST and incubated at room temperature with secondary antibodies for 1 hour. Unbound secondary antibodies were removed with 3x10 minute washes in TBST prior to development by chemiluminescence (ECL) or scanning using the Odyssey® Infrared Imaging System (Licor), dependent on the secondary antibody used (shown in Tables 2.4).

2.2.18.1. Chemiluminescence

Secondary antibodies covalently linked to horseradish peroxidase (HRP) can be visualized using the ECL Detection Kit (Amersham). The HRP enzyme catalyses the oxidation of luminal, and in the presence of an enhancer (phenol), results in the emission of light. The treated membranes were incubated in the ECL detection reagent for 1 min before exposure of the membranes to the CL-Xposure™ Film (Thermo Scientific) for various times (10 secs to 15 minutes) and development in an automatic film developer (X-OGRAPH Compact X4-XOgraph, Gloucester, UK).

1.2.1.18.2 Quantification

The images of blots were scanned using an Epson Perfection 2400 Photo scanner and quantified using the Aida Image Analyser software version 3.28 (Raytest).

2.2.18.2. Odyssey® Infrared Imaging System

Infrared scanning allows the quantification of secondary antibodies labelled with infra-red dyes. The scanner uses two separate channels, 700 and 800 nm, and as such allows analysis of both phosphorylated and total levels of the same protein on the same membrane as long as the primary antibodies are from different species to permit use of distinct secondary antibodies. Once the membranes had been washed for the final time, they were allowed to dry before scanning using both the 700 and 800 nm channels. Quantification was performed using the Odyssey Application Software version 3 (Licor).

2.2.19. Stable Transfections

Stable transfection occurs when the recombinant vector inserts into the genome of the host cell in a position that results in expression of the inserted gene or regulation of an inserted gene promoter. This can be achieved by culturing cells in a selective media, such that only those transfected cells, containing the resistance gene, will grow and non transfected cells will die. Similarly, as transiently transfected cells throw out the recombinant construct (or it becomes diluted during cell division) they become sensitive to the selection media. Prolonged resistance is only achieved by stable insertion of the DNA into the host genome in a fashion that results in equal transmission during cell division. Transfections were performed using the calcium phosphate technique (Sutherland, 1999).

2.2.19.1. Stable transfection of H4IIe cells

H4IIe cells are resistant to DNA uptake, with an average transfection efficiency of <1%. Cells were cultured in T75 flasks until 50% confluent (approximately $5x10^6$ cells). 2 µg DNA (2-4 µl of 0.5-1 µg/µl) was precipitated in sterile 14 ml polypropylene tubes containing 250 µl 1X BES (Table 2.1), 216-218µl RNAse free water and 30µl 0.15 M $CaCl_2$ for 20 minutes with gentle agitation every 2-3 minutes. During the incubation period, 1 flask of cells H4IIe cells per transfection were isolated by trypsinisation, cells washed in 5 ml sterile PBS and centrifuged again for 4 minutes at 3,500 rpm. The PBS was aspirated, and the precipitated DNA mixture added to the cells which were mixed by flicking. The DNA/cell mixture was incubated at room temperature for a further 20 minutes to allow the DNA complex to bind to cell membranes. 0.5 ml of the cell mixture was placed in 10 cm dishes containing 10 ml of complete media and allowed to settle for 4 hours in a 5% CO_2 water saturated incubator. Following this, the media was aspirated and the cells were shocked with 20% (v/v) DMSO in complete media for 5 minutes to facilitate DNA uptake into cells. The media was aspirated, the cells gently washed twice in complete media and 10 ml complete media added to the dishes which were then returned to a 5% CO_2 water saturated incubator for 48 hours. Media was aspirated and replaced with complete media containing 500 µg/ ml G418, and this was replaced with fresh G418 media every 3-4 days.

2.2.19.2. Isolating colonies of stably transfected H4IIe cells

After 2 weeks in selection media, individual colonies of potential stable transfectants were identified. Media was aspirated from the cells which were then washed in 5 ml sterile PBS. Colony isolation rings (Sigma) were smeared with sterile Vaseline® petroleum jelly. These were placed over the colonies and 100 µl trypsin/EDTA added to the centre of each ring. The plates were returned to the incubator for 5 minutes after

Primary Antibody	Molecular Weight (kDa)	Dilution	Species	Company	Catalogue Number
Phospho-PKB (Ser473)	60	1:1000	Rabbit	CST	#9271
Phospho- PKB (Thr 308)	60	1:1000	Rabbit	CST	#9275
PKB	60	1:1000	Rabbit	CST	#9272
PKB (pan) (40D4)	60	1:1000	Mouse	CST	#2920
Phospho-AMPK (Thr172)	66	1:1000	Rabbit	CST	#2535
Total AMPK					
Phospho-ACC (Ser79)	265	1:1000	Rabbit	CST	#3661
Total p42/p44	42/44	1:1000	Rabbit	CST	#9102
Phospho-p42/44 (Thr202/Tyr204)	42/44	1:1000	Rabbit	CST	#9101S
Phospho-S6 (Ser240/244)	32	1:1000	Rabbit	CST	#2215
Total S6 (5G10)	32	1:1000	Rabbit	CST	#2217
Total IRS-1	165	1:1000	Rabbit	MP	06-248
Phospho-Tyr (4G10)	N/A	1:25000	Mouse	MP	05-321
B-Actin	42	1:5000	Rabbit	Sigma	#A2066
Phospho-IRS-1 (Ser307)	180	1:1000	Rabbit	CST	#2381
Phospho GSK-3α/β (Ser21/9)	51/46	1:1000	Rabbit	CST	#9331
Total GSK-3β	46	1:1000	Mouse	BD	610202
Phospho FOXO1 (Ser256)	82	1:1000	Rabbit	CST	#9461
Total FOXO1	82	1:1000	Sheep	DSTT	N/A
Phospho SMC (Ser966)	160	1:1000	Ra bbi t	Bethyl	A30-050A
Total SMC1	160	1:1000	Rabbit	Bethyl	A300-055A

Table 2.4 List of Primary antibodies

Secondary Antibody	Dilution	Species	Company	Catalogue Number
HRP Conjugated Rabbit Anti-Sheep	1:5000	Rabbit	TS	#31480
HRP Conjugated Goat Anti-Rabbit	1:5000	Goat	TS	#31480
HRP Conjugated Rabbit Anti-Mouse	1:5000	Rabbit	TS	#31480
Alexa Fluor® 680 Goat Anti-Mouse IgG (H+L)	1:5000	Goat	Invitrogen	A-21057
IRDye800® Conjugated Goat Anti-Rabbit IgG (H&L)	1:5000	Goat	Rockland	611-132-122

Table 2.5 List of secondary antibodies

which cells were dissociated by quickly pipetting up and down. The cell suspension was transferred to the single well of a 96 well plate containing 250 µl of complete media to neutralise the trypsin. These were returned to a 5% CO_2 water saturated incubator until confluent. Once confluent, the cells were passaged into vessels of increasing size in selection media until adequate numbers of cells were obtained for analysis and freezing.

2.2.20. Transcriptional Reporter Assays

Reporter assays are a common way of analyzing gene transcription in cells. Production of the reporter protein is directly proportional to the activation of the gene promoter driving the reporter gene and can be used to measure the regulation of complete gene promoters. In this thesis, the reporter constructs have been stably transfected into H4IIe cells.

2.2.20.1. Luciferase Assay

Luciferase is an enzyme present in a number of bioluminescent organisms. Firefly (*Photinus pyralis*) luciferase is commonly used in molecular biology and is the reporter produced by pGL4.17. This enzyme oxidizes luciferin in the presence of ATP and magnesium ions to produce oxyluciferin, carbon dioxide and pyrophosphate ions as well as light in the range of 550-570 nm.

Prior to the luciferase assay, media was aspirated and the cells washed once in ice cold PBS. Cells were lysed in 150 µl of Cell Culture Lysis Buffer (Promega) and disrupted with a cell scraper. The contents of each well were transferred to a clean microcentrifuge tube and placed on ice. Samples were centrifuged at 13,000 rpm, 15 minutes 4°C and the supernatant transferred to a clean microcentrifuge tube. The Luciferase Assay Reagent was reconstituted by adding 10ml Luciferase Assay Buffer

(Promega) to 1 vial lyophilized Luciferase Assay Substrate (Promega). For each sample 100 µl of Luciferase Assay Reagent was added to a clean 5 ml clear plastic tube followed by 20 uL of lysates. This was mixed by briefly vortexing and then reading with a luminometer (Berthold Lumat LB9507 for 2 seconds. Each sample was measured in duplicate and the samples were normalized to total protein in each lysate (Chapter 2.2.14.1).

2.2.21. Collection and processing of human serum

Prior to attendance for serum donation, volunteers were fasted for 12 hours. On arrival, they underwent anthropomorphometry prior to venepuncture and collection of 229 ml blood. Blood to be analysed by the Clinical Biochemistry Department, Ninewells Hospital Dundee was allocated as per Table 2.6. In addition, 8.5 ml whole blood was collected in a BD P100 collection tube to generate plasma. The tube was inverted gently 10 times and then centrifuged at 3000 rpm for 5 minutes at room temperature. A maximum of 8 x 0.5 ml aliquots were transferred to clean 1.5 ml tubes, the samples snap frozen and stored at -80°C. Serum was prepared for analysis of inflammatory markers from 5 ml of whole blood collected in a BD SST™ Tube with Silica Clot Activator. This was allowed to stand for 30 minutes at room temperature then centrifuged at 3000 rpm for 10 minutes. A maximum of 2x 0.5 ml and 8x 0.1 ml aliquots were transferred to clean storage tubes, snap frozen and stored at -80°C. To obtain additional serum for cell culture, 200 ml whole blood was collected in 20x BD Serum Tubes with Increased Silica Act Clot Activator. These were allowed to stand at room temperature for 1 hour before centrifugation at 3000 rpm for 10 minutes. All serum was transferred to sample tubes in 10 ml aliquots, snap frozen and stored at -20 ◦C.

Collection Tube	Volume	Assays Requested
Fluoride	4 ml	Glucose
EDTA	4 ml	HbA1c
Clotted Serum	5 ml	Alanine Aminotransferase, Bilirubin, Alkaline Phosphatase, Albumin, Aspartate Transaminase, Total Cholesterol, HDL Cholesterol, LDL Cholesterol

Table 2.6 Collection tubes and fate of collected blood from volunteers

Plasma and serum was sent to the TMRC, Ninewells Hospital, Dundee for assay of Leptin, Adiponectin, CRP and TNFα by sandwich ELISA (Logie et al., 2010).

2.2.22. Statistical Analysis

Logistical regression and contingency table analysis was performed using SPSS statistics 17.0 (IBM, USA). Comparison data between groups was analysed by students t-test using Microsoft Excel 2003 (Microsoft, USA). Graphical data is shown as mean +/- standard error of the mean unless otherwise stated.

Chapter 3. Development and Validation of a Cell Based Model of Insulin Resistance.

3.1. Introduction

Insulin resistance is a continuous variable inversely correlated with the risk of developing diabetes as well as the associated morbidity and mortality. Developing strategies and therapeutics to improve insulin sensitivity is a global healthcare priority. For this to be achieved, we must first understand what factors dictate the insulin sensitivity of a cell, organ and individual. Insulin sensitivity is affected by multiple factors. For example adiponectin is reduced in obese individuals (Hotta et al., 2001) and the administration of adiponectin reduces hepatic glucose output in mouse models of obesity (Yamauchi et al., 2001). Other factors such as fatty acids, (Massillon et al., 1997) TNF-α (Uysal et al., 1997) and IL-6 (Mooney et al., 2001) which are higher in individuals with obesity have all been found to have negative effects on insulin signalling.

Our laboratory found that culturing rat hepatoma cells in serum from rats with diet induced obesity results in a 10 fold decrease in insulin sensitivity (Logie et al., 2010). A similar effect was obtained by culturing rat hepatoma cells in the serum from leptin receptor deficient obese rats (Logie et al., 2010). This effect is reversible both with the reversion of culture conditions to standard FCS and also by culturing cells in the presence of the common anti-diabetic drugs metformin or pioglitazone (Logie et al., 2010). Taken together it is reasonable to assume that one or more components altered in the serum from the obese animals (potentially adiponectin, fatty acids, TNF-α or IL-6) was having an effect on insulin sensitivity.

Current animal models of type 2 diabetes are often inbred strains with one or more genetic mutations that predispose the offspring to readily develop obesity, insulin resistance and hyperglycaemia (see Chapter 1.13). These models, although useful, do

not represent the changes seen in the majority of human disease where very few cases of T2DM develop due to a monogenetic disorder.

I aimed to test whether serum from obese human diabetics would induce insulin resistance in H4IIe cells, in a similar manner to serum from obese rodents. In this chapter I describe the clinical parameters of volunteers recruited to supply the serum for culture with emphasis on the differences between them. I go on to describe the specific effects that serum from obese diabetic volunteers (in direct comparison with serum from lean individuals) has on insulin regulation of gene transcription and cell signalling. Finally, I search for biomarkers associated with the generation of the insulin resistance.

3.2. Results

3.2.1. Characterisation of study groups

Twenty lean male non-diabetic volunteers (controls) and twenty male obese diabetic individuals (cases) were recruited to the study. Controls were required to be euglycaemic and have no first degree relatives with diabetes and cases were all diagnosed as having diabetes according to WHO criteria (Alberti and Zimmet, 1998). None of the volunteers in either group were taking antihyperglycaemic medication at any point prior to the commencement of the study. Smokers and those taking the fibrate group of drugs were also excluded. Full inclusion and exclusion criteria are shown (Appendix 1). Volunteers were fasted for 12 hours prior to assessment. Basic clinical measurements are listed in Table 3.1. Each volunteer donated 290 ml of blood. The blood was processed to obtain serum for tissue culture (Chapter 2.2.21). Plasma was collected for insulin, CRP, adiponectin, leptin and TNF-α (performed by Dr Jeff Brady, TMRC), and for assessment of bilirubin, alanine amino transferase, aspartate aminotransferase, alkaline phosphatase, albumin, glucose, HbA1c, total HDL and LDL cholesterol, and triglyceride levels (performed by the Clinical Biochemistry Department, Ninewells Hospital). HOMA-IR was calculated from measured fasting insulin and glucose levels (See Chapter 1.2).

Age, weight, height, waist circumference, BMI, hip circumference, waist:hip ratio, systolic blood pressure, pulse, HOMA-IR, Insulin, CRP, and leptin were all significantly higher in the cases compared to the controls and as expected, adiponectin was significantly lower in the cases. Surprisingly total cholesterol, LDL cholesterol and triglycerides were significantly lower in the cases. On further investigation, all volunteer cases were found to be receiving HMGCoA reductase inhibitors which accounts for this unexpected difference. Therefore I have collected two phenotypically

	Controls (n=20)	Cases (n=20)	P value
Age (Years)	50.85 (5.96)	56.7 (3.70)	0.001
Weight (Kg)	77.51 (9.12)	106.67 (16.55)	<0.0001
Height (cm)	181.1 (7.45)	174.7 (6.81)	0.007
Waist (cm)	88.25 (6.47)	117.95 (12.85)	<0.0001
BMI (kg/m^2)	23.45 (1.32)	34.95 (5.00)	<0.0001
Hip (cm)	99.7 (3.88)	115.95 (8.06)	<0.0001
Waist:Hip ratio	0.88 (0.06)	1.02 (0.05)	<0.0001
Systolic BP (mmHg)	126.1 (16.06)	139.6 (17.97)	0.017
Diastolic BP (mmHg)	78 (9.07)	83.25 (8.01)	0.059
Pulse (bpm)	61.4 (10.22)	74.75 (13.22)	0.001
Total cholesterol	5.50 (0.93)	4.42 (0.74)	<0.0001
LDL (mmol/L)	3.39 (0.84)	2.37 (0.54)	<0.0001
HDL (mmol/L)	1.55 (0.38)	1.19 (0.35)	0.006
Trigs (mmol/L)	1.27 (1.09)	2.14 (1.41)	0.039
HOMA- IR *	0.75 (0.45-1.26)	2.15 (1.50 -3.08)	<0.0001
Insulin *	5.69 (3.35-9.68)	15.11 (10.26 – 22.24)	<0.0001
CRP *	532.57 (189.49-1496.84)	2345.40 (1117.04-4924.55)	<0.0001
Adiponectin *	7033.40 (4301.97-11499.10)	4205.06 (2591.94-6822.13)	0.002
Leptin *	1198.39 (188.27-7627.97)	12976.36 (7142.59-23574.93)	<0.0001
TNF-α *	1.13 (0.74-1.74)	1.08 (0.79-1.47)	0.687
ALT (U/l)	25.9 (13.80)	38.7 (11.32)	0.003
AST (U/l)	28 (6.37)	26.3 (6.98)	0.43
Bilirubin (µmol/l)	14.35 (8.05)	12.35 (5.19)	0.36
Alkaline Phosphatase (U/l)	66.2 (14.28)	77.9 (13.41)	0.0009
Albumin (g/l)	46.8 (3.04)	45.6 (2.23)	0.16

Table 3.1 Baseline characteristics of the human volunteers mean (SD) * Non-normally distributed data presented as geometric means (range of SD)

defined cohorts to provide serum to test for the presence of components related to diabesity that generate insulin resistance in cell culture.

3.2.2. The insulin sensitivity of the PEPCK gene promoter changes in cells grown in serum from patients with diabesity.

H4IIe cells were cultured for 3 weeks in DMEM containing serum (at 5%) from each individual (20 controls and 20 cases). During the culturing and analysis I was blinded to the case or control status of each sample. After 3 weeks, all 40 pools of cells were fasted for 3 hours prior to stimulation with the synthetic glucocorticoid dexamethasone (500 nM) and the second messenger cAMP (100 μM 8-CPTcAMP) in the presence or absence of increasing concentrations of insulin. Total cellular RNA was extracted and cDNA synthesised before assessment of PEPCK and actin mRNA levels by Taqman analysis. Data is shown as the ratio of PEPCK to actin mRNA (Figure 3.1). As expected, dexamethasone and cAMP stimulated the production of PEPCK mRNA, and this was dominantly repressed by the presence of insulin. Cells cultured in serum from cases exhibited insulin resistance relative to cells grown in control serum, as shown by the reduced ability of 0.1 and 0.5 nM insulin ($p<0.0001$ for both) to prevent induction of PEPCK (Figure 3.1). There was no difference in the basal levels of PEPCK (Figure 3.1). There was no difference in fold induction in PEPCK expression by dexamethasone and cAMP (Figure 3.1 and 3.2 A). However, the percentage repression of PEPCK by 0.1 nM insulin was reduced from 113.4 ± 12.4% (i.e. below basal PEPCK levels) in the control cells to 53.7 ± 24.7% in cells grown in serum from the cases (Figure 3.2 B). Similarly, the percentage repression of PEPCK by 0.5 nM insulin was reduced from119.5 ± 11.1% in the control cells to 78.9 ± 13.6% in the cells cultured for 3 weeks in serum from obese diabetics (Figure 3.2 C). The basal level of PEPCK mRNA (serum free for 3h) of the cells grown in diabesity serum is generally lower than control cells,

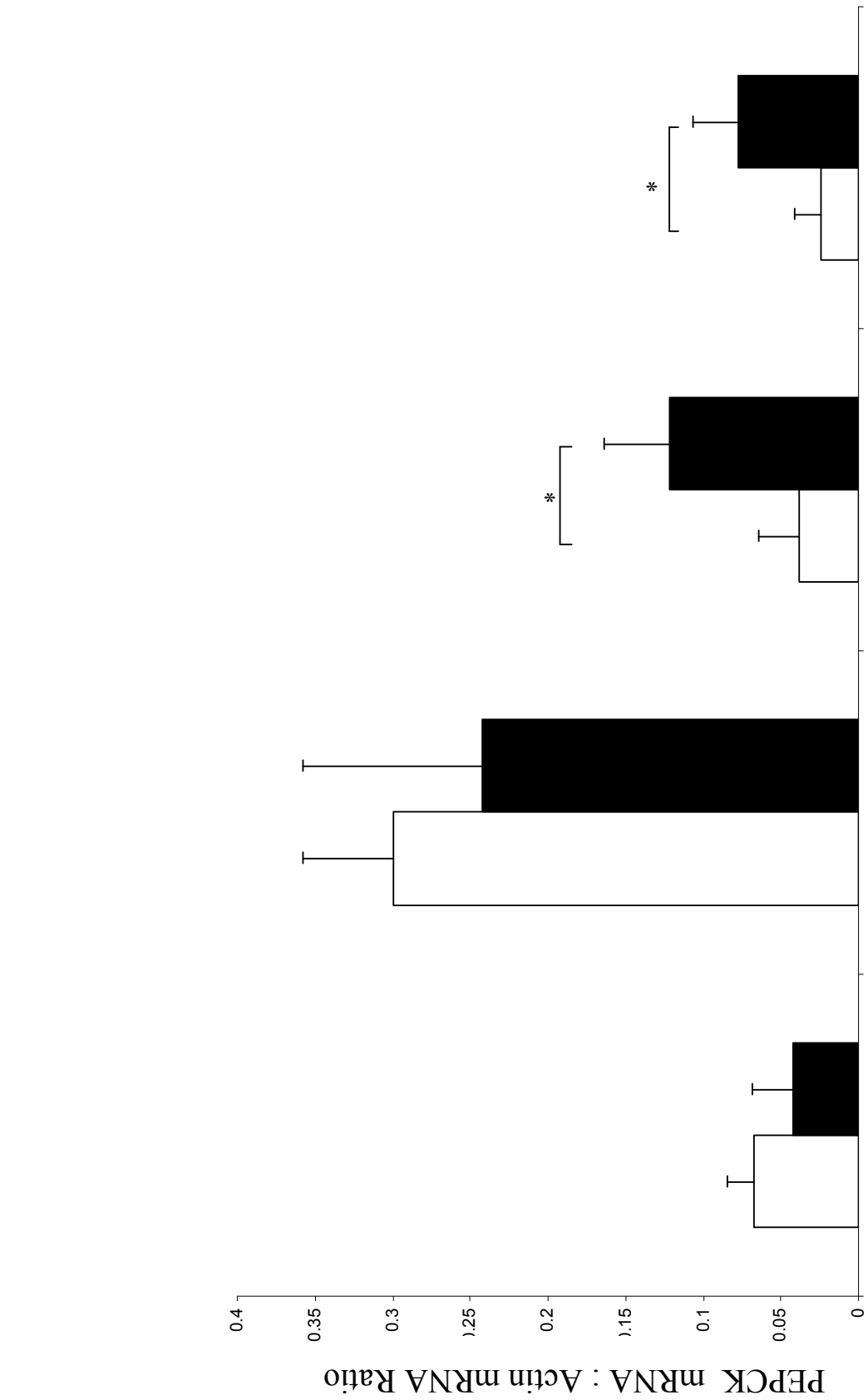


Figure 3.1 Comparison of the effect of growing cells in serum from obese or lean humans on insulin regulation of PEPCK mRNA. H4IIe cells were grown for 3 weeks in 5% serum collected from 40 human volunteers, 20 who were obese and diabetic (cases), 20 who were lean and non-diabetic (controls). After 3 weeks in the serum the 40 different pools of cells were serum starved for 3 h prior to 3 h exposure to dexamethasone (500 nM), 8CPT-cAMP (0.1 mM) and insulin as indicated, and RNA was isolated. PEPCK mRNA levels (average +/- Std dev) are presented (n = 20, assayed at least in duplicate for each condition) after correction for actin mRNA. White bars = controls, Black Bars = Cases * = $p<0.0001$

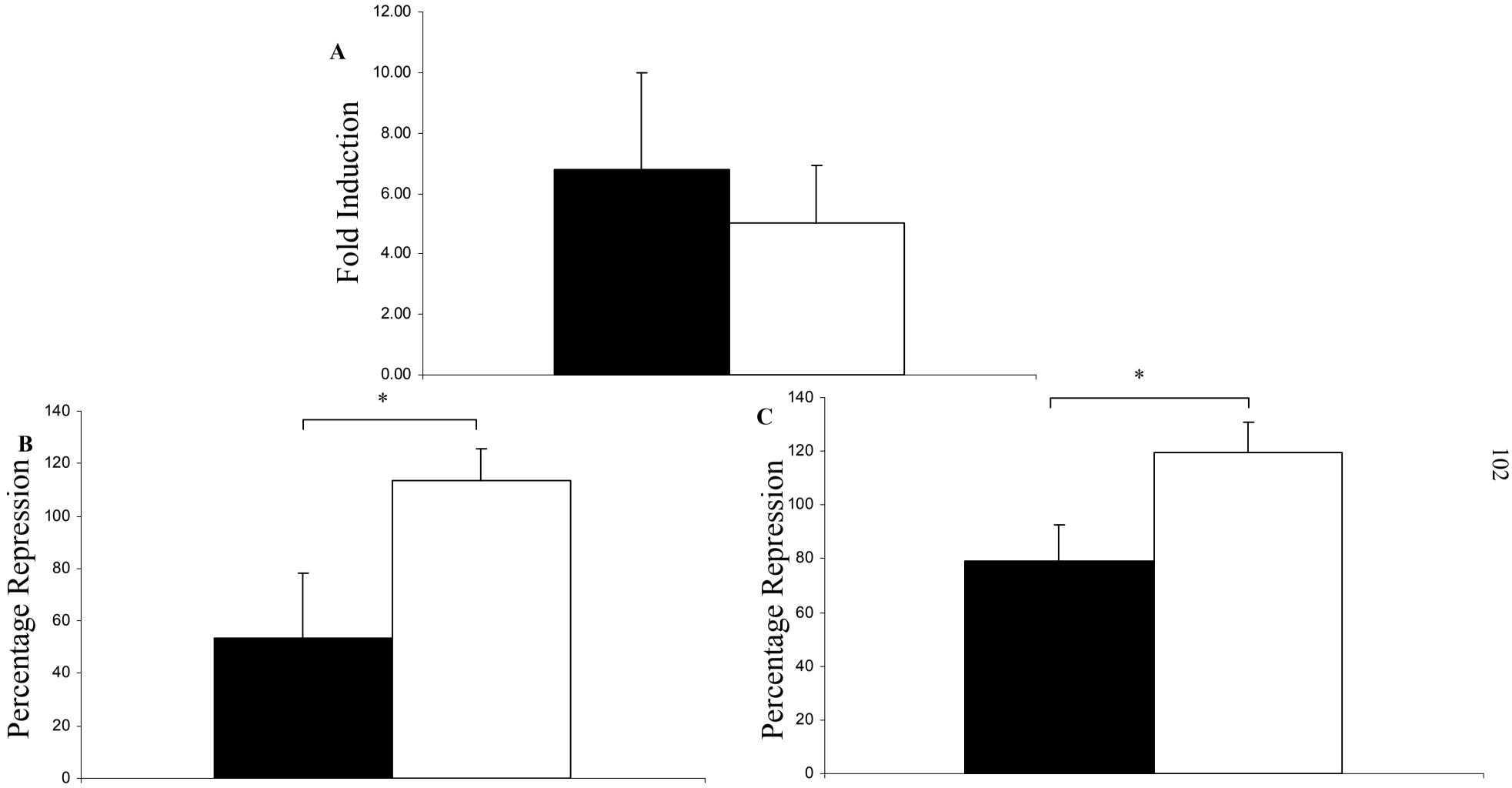


Figure 3.2 Mean changes in PEPCK gene expression for each group (A) Mean fold induction from basal with the addition of Dexamethasone and cyclic AMP, (B) Mean percentage repression with 0.1 nM insulin from maximal stimulation with Dexamethasone and cyclic AMP, (C) Mean percentage repression with 0.5 nM insulin from maximal stimulation with Dexamethasone and cyclic AMP. Black bars = cases, white bars = controls. Error bars indicate standard deviation. * = $p<0.0001$ (n = 20, assayed at least in duplicate for each condition)

although this doesn't reach significance as a group (Figure 3.1). In fact, 70% of the cases have levels below the lowest basal level of PEPCK in the controls. However, the remaining 30% are scattered throughout the range of basal PEPCK levels in the experimental group (Figure 3.3). Similarly, the induction in PEPCK mRNA levels by dexamethasone and cAMP is not significantly different between cases and controls. Nevertheless, 50% of cases have a higher fold induction than all of the controls (Figure 3.4), and this means that the actual PEPCK mRNA levels in cells treated with dexamethasone and cAMP are very similar between the two groups (slightly lower basal but slightly higher induction of cases). The highest fold induction was observed in the cells with the lowest basal PEPCK mRNA (those grown in serum from case 16).

There is clearly a reduced response of PEPCK to insulin in almost all 20 pools of cells grown in diabesity serum compared to cells grown in control serum (Figure 3.5). Namely, in 18 out of the 20 groups of cells in serum from cases PEPCK is repressed by 0.1 nM insulin by less than 80%, whereas PEPCK is repressed greater than 80% in all but one group of cells grown in control serum (Figure 3.5). The cells with the lowest basal and highest induction of PEPCK mRNA (grown in Case 16) had one of the poorest responses to insulin. Conversely, the cell with the highest basal and the second lowest induction of PEPCK mRNA (grown in control 17) had the best insulin response. Likewise, PEPCK is reduced below basal by 0.5 nM insulin in 18 out of 20 of the control cells, but in only 1 out of 20 of the cells exposed to case sera (Figure 3.6). In fact, the top 42.5% and 45% responses to 0.1 and 0.5 nM insulin respectively are from cells grown in control sera, and conversely the lowest 42.5% responses to insulin are cells grown in sera from cases (Figures 3.5 and 3.6). These data indicate that culturing H4IIe cells in the serum from obese diabetics can induce insulin resistance as measured by a significantly reduced repression of PEPCK mRNA by insulin.

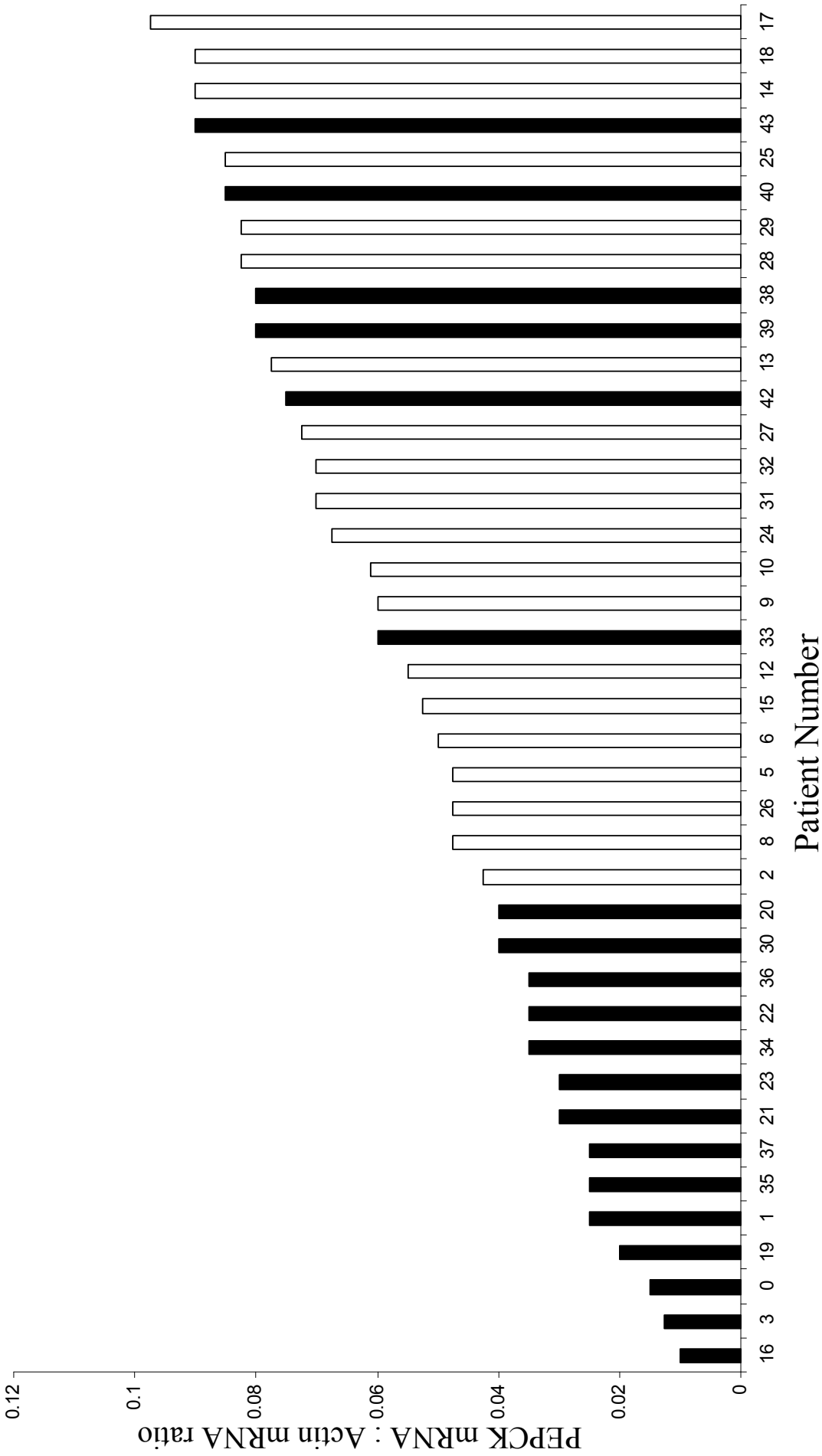


Figure 3.3 Basal levels of PEPCK in experimental patient group. All subjects are shown and have been ranked in ascending order of basal PEPCK levels. Black bars = cases, white bars =controls.

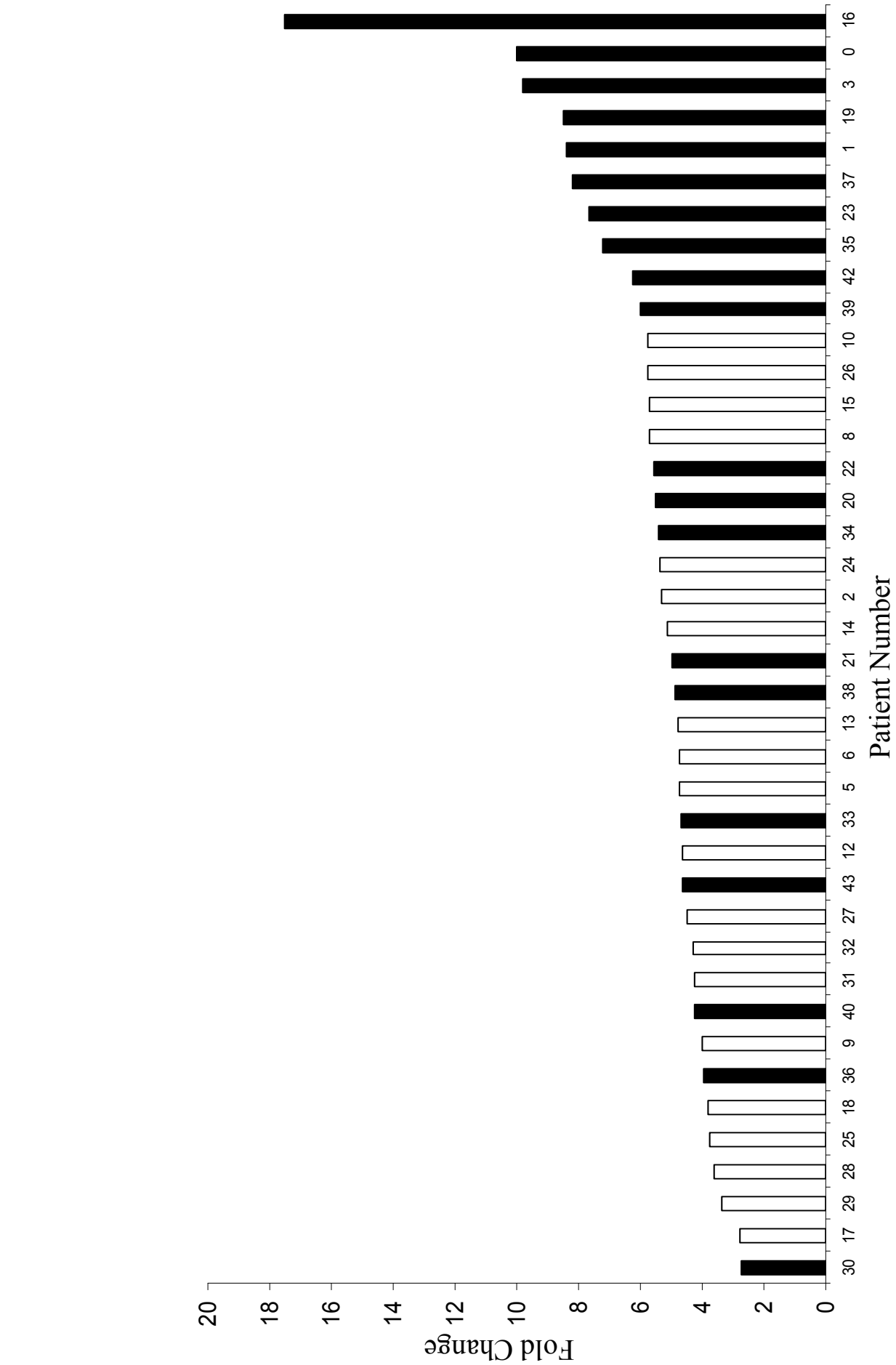


Figure 3.4 Fold change in PEPCK expression above basal with the addition of Dexamethasone and cyclic AMP. All subjects are shown and have been ranked in ascending order of fold induction. Black bars = cases, white bars =controls.

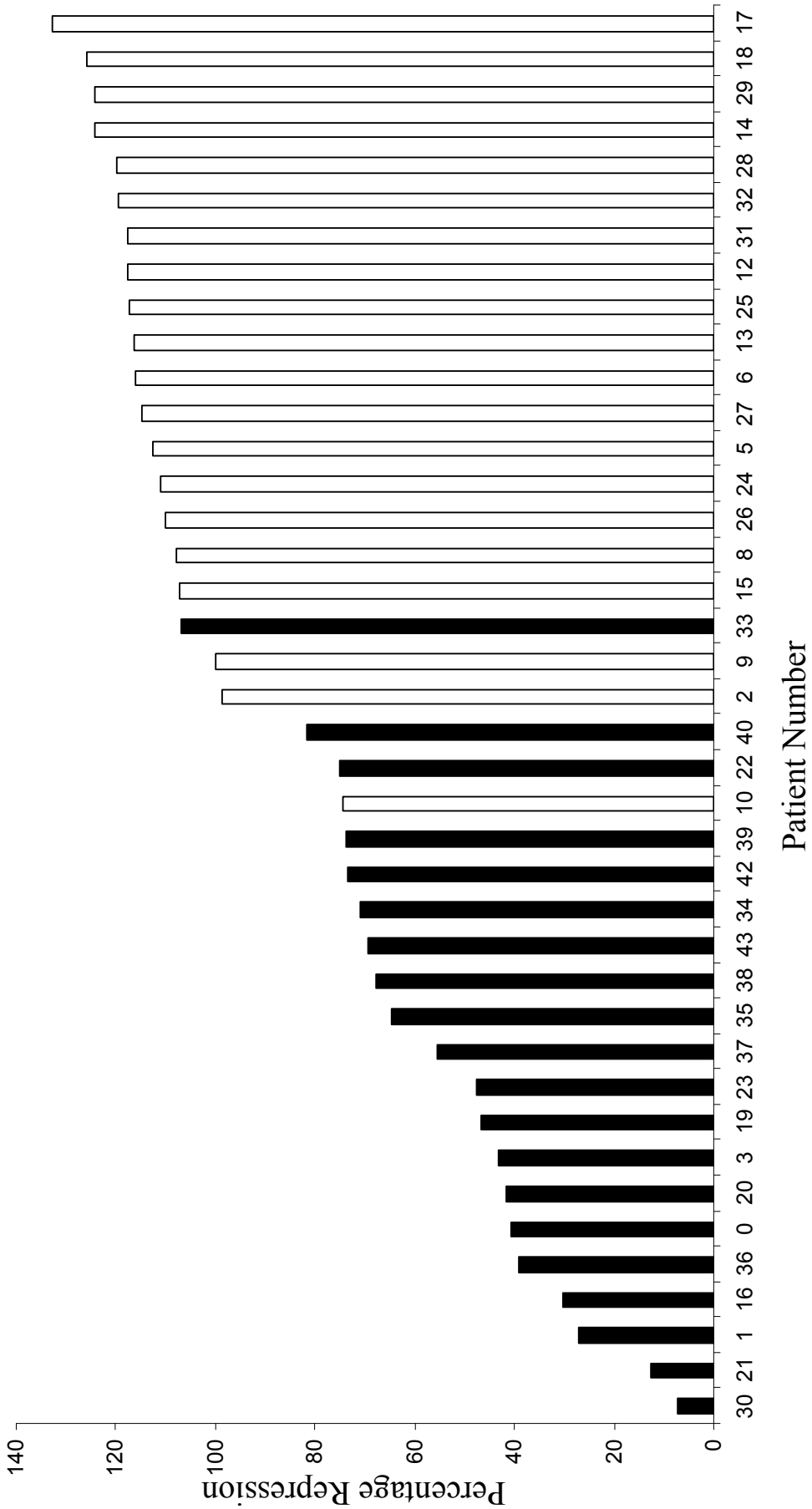


Figure 3.5 Percentage repression of PEPCK expression below maximal stimulation by dexamethasone and cAMP by the addition of 0.1 nM Insulin. All subjects are shown and have been ranked in ascending order of percentage repression. Black bars = cases, white bars =controls.

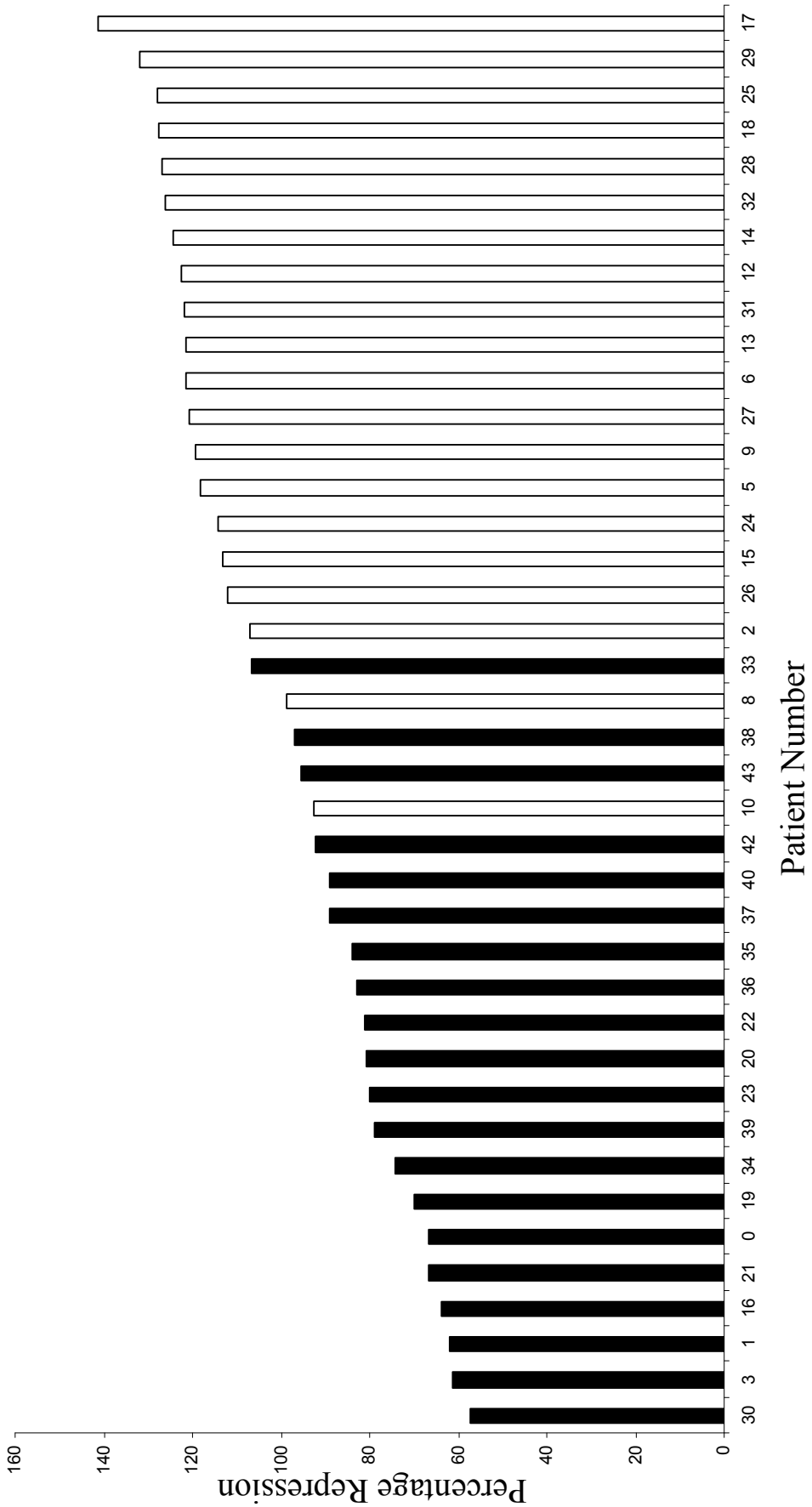


Figure 3.6 Percentage repression of PEPCK expression below maximal stimulation by dexamethasone and cAMP by the addition of 0.5 nM insulin. All subjects are shown and have been ranked in ascending order of percentage repression. Black bars = cases, white bars =controls.

3.2.3. Identification of serum factors responsible for the generation of insulin resistance.

There are significant differences between the levels of several serum factors in cases and controls but interestingly the concentration of TNFα is similar in each group (Table 3.1). In an unbiased effort to find factors that may be responsible for the generation of insulin resistance, linear regression modelling was performed (Table 3.2). The aim was to see if the effect of insulin on PEPCK repression was independent of differences in the baseline data between groups. Non-normally distributed data was log transformed to allow for robust data analysis. The first model used age, BMI and case/control status as covariates. The second model added each of the other covariates of glucose, HbA1c, insulin, HOMA-IR, triglycerides, leptin, adiponectin, TNF-α, CRP, systolic blood pressure, diastolic blood pressure and pulse in turn. Modelling did not include any serum lipids due to the confounding factor of HMGCoA reductase inhibitor use. Height, weight and waist circumference, hip circumference and waist:hip ratio were also excluded as they are very strongly correlated with BMI.

Analysis of covariance showed that case/control status predicted the ability of 0.1 nM insulin to repress PEPCK ($p<0.001$) (Table 3.2). This correlation is not diminished when data is corrected for the effects of BMI (Table 3.2). Addition of further covariates of glucose, HbA1c, insulin, HOMA-IR, triglycerides, leptin, adiponectin, TNF-α, CRP, systolic blood pressure, diastolic blood pressure and pulse did not eliminate the strong correlation of diabetes status with the repressive ability of insulin (Table 3.2). This data suggests that no single clinical or biochemical factor measured can explain the percentage repression of PEPCK by insulin in an individual.

		Coefficient p-value			
		Case-Control	**Age**	**BMI**	**Other**
Model 0		<0.001	-	-	-
Model 1		0.003	0.41	0.25	-
Model 2	**Glucose**	0.029	0.45	0.30	0.06
	HbA1c	0.012	0.42	0.29	0.56
	Insulin	0.003	0.38	0.20	0.52
	HOMA-IR	0.003	0.38	0.21	0.57
	Triglycerides	0.004	0.44	0.28	0.92
	Leptin	0.005	0.41	0.30	0.92
	Adiponectin	0.003	0.46	0.22	0.65
	TNF-α	0.005	0.40	0.17	0.29
	CRP	0.002	0.52	0.18	0.39
	Systolic BP	0.003	0.49	0.29	0.77
	Diastolic BP	0.004	0.47	0.28	0.63
	Pulse	0.004	0.42	0.26	0.94

Table 3.2 Linear regression models. The dependent variable is percent repression of PEPCK by 0.1 nM insulin. Model 0 is the comparison by case/control status. Model 1 adds age and BMI as covariates. Model 2 adds each additional biochemical parameter to model 1. The p-values represent the coefficient p-values in the model.

Although it was not possible to find a single biomarker by logistic regression, I attempted to correlate percentage repression of PEPCK by 0.1 and 0.5 nM insulin with levels of particular serum factors (Figures 3.7 -3.11). As obesity increases, so leptin levels increase. It is clear that those subjects with the highest leptin levels are cases, and that they also have the lowest levels of insulin repression. However, there is no simple linear correlation between fasting leptin levels and the level of PEPCK repression (Figure 3.7A and 3.7B). However if the data is split into quartiles by percentage repression of PEPCK by insulin, that is, <50%, 50-75%, 75.1-110% and >110% for 0.1 nM insulin and <75%, 75-100%, 100.1-120% and >120% for 0.5 nM insulin, there is a correlation between the mean leptin of each group and PEPCK repression (Figure 3.7 C and D, r^2 value of 0.85 with 0.1 nM insulin and 0.94 with 0.5nM insulin). However, there is still sufficient variation in leptin concentration within these quartiles that would not permit the use of leptin concentration alone as a simple biomarker of insulin sensitivity (Figure 3.7C and D), at least as measured by repression of PEPCK mRNA in this cell model.

As expected cases generally have a lower HOMA-IR compared to controls. Nevertheless, there is no correlation between HOMA-IR and the level of insulin resistance seen in the cell line (Figure 3.8A and B). As with leptin there is a correlation between quartile of PEPCK repression and mean HOMA-IR (r^2 value of 0.99 and 0.91 for 0.1 and 0.5 nM insulin respectively Figure 3.8 C and D). Insulin levels were significantly different between cases and controls. Despite this, there is no linear association between plasma insulin levels and the effect of the sera on the response of H4IIe cells to 0.1 or 0.5 nM insulin (Figure 3.9A and B). There is a strong correlation between fasting insulin levels and the effect of sera on the insulin sensitivity of the cells when the latter is grouped as quartiles (r^2 value of 0.99 and 0.91 respectively for

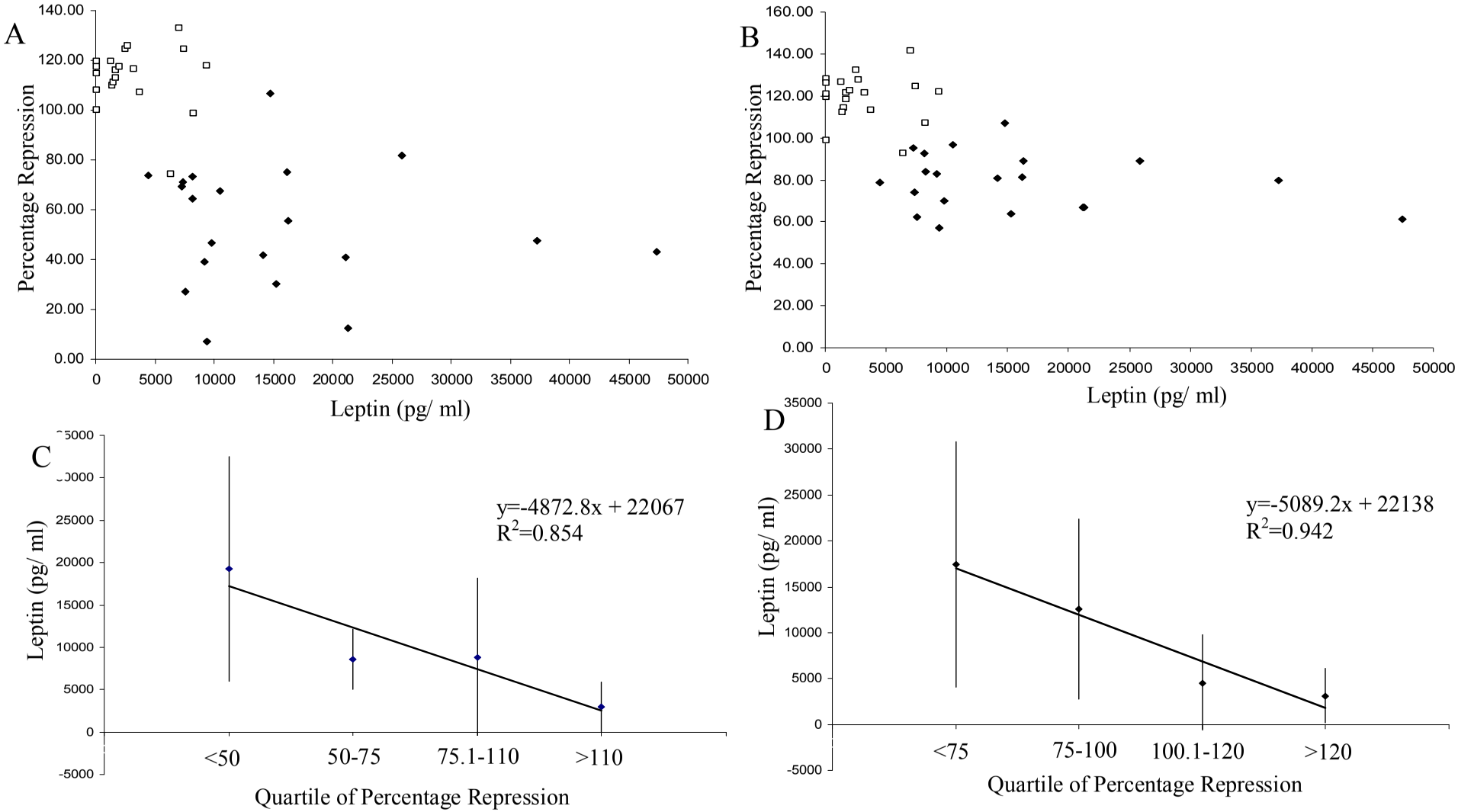


Figure 3.7 Scatter plot of Leptin and percentage repression of PEPCK by (A) 0.1 nM insulin, (B) 0.5 nM Insulin All experimental subjects shown. Hollow squares= controls black diamonds = cases. (C) Quartile of percentage repression by 0.1 nM insulin against mean leptin level (D) Quartile of percentage repression by 0.5 nM insulin against mean leptin level Data shown as Mean +/- SD

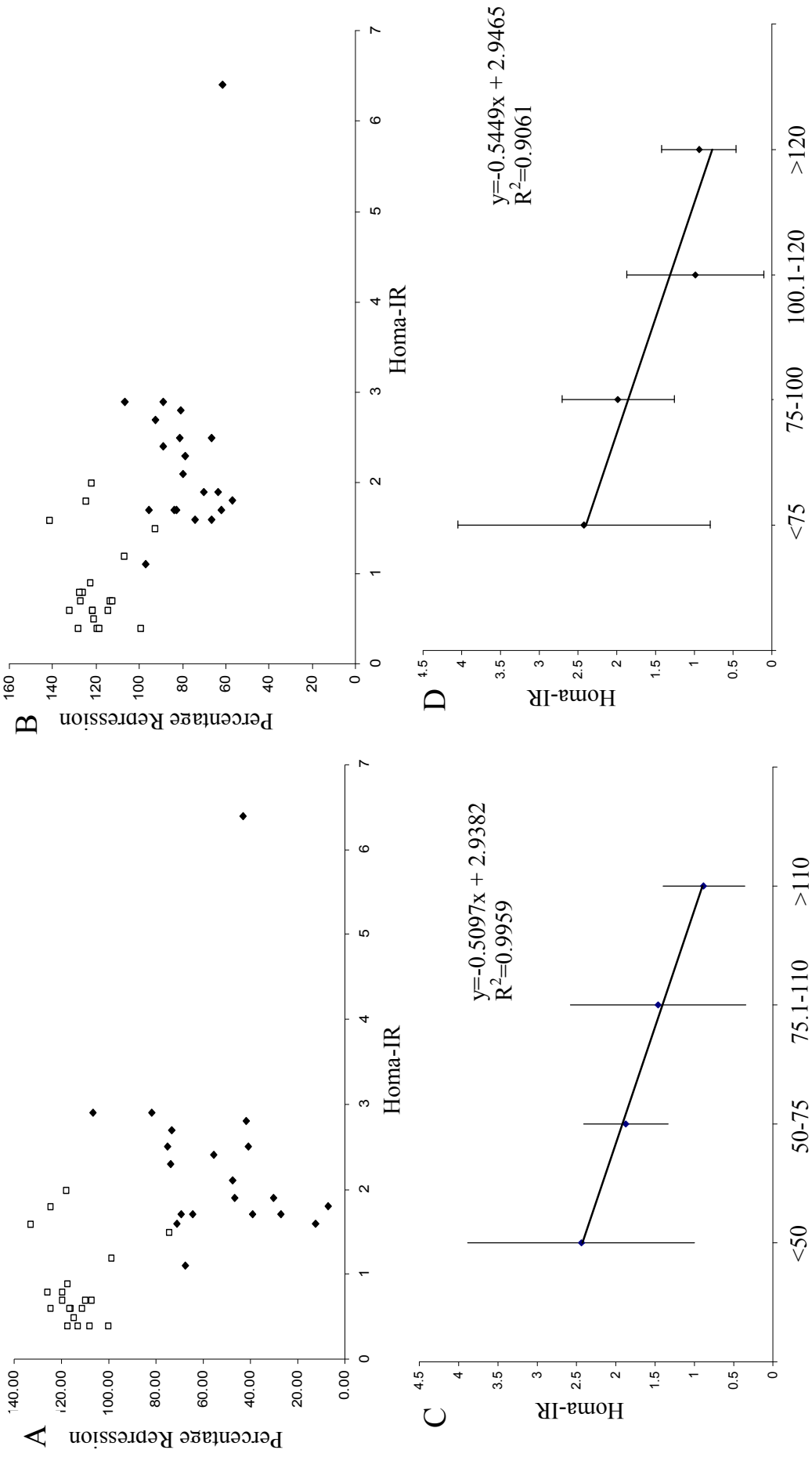


Figure 3.8 Scatter plot of HOMA-IR and percentage repression of PEPCK by (A) 0.1 nM insulin, (B) 0.5 nM Insulin. All experimental subjects shown. Hollow squares= controls black diamonds = cases. (C) Quartile of percentage repression by 0.1 nM insulin against mean HOMA-IR level (D) Quartile of percentage repression by 0.5 nM insulin against mean HOMA-IR level Data shown as Mean +/- SD

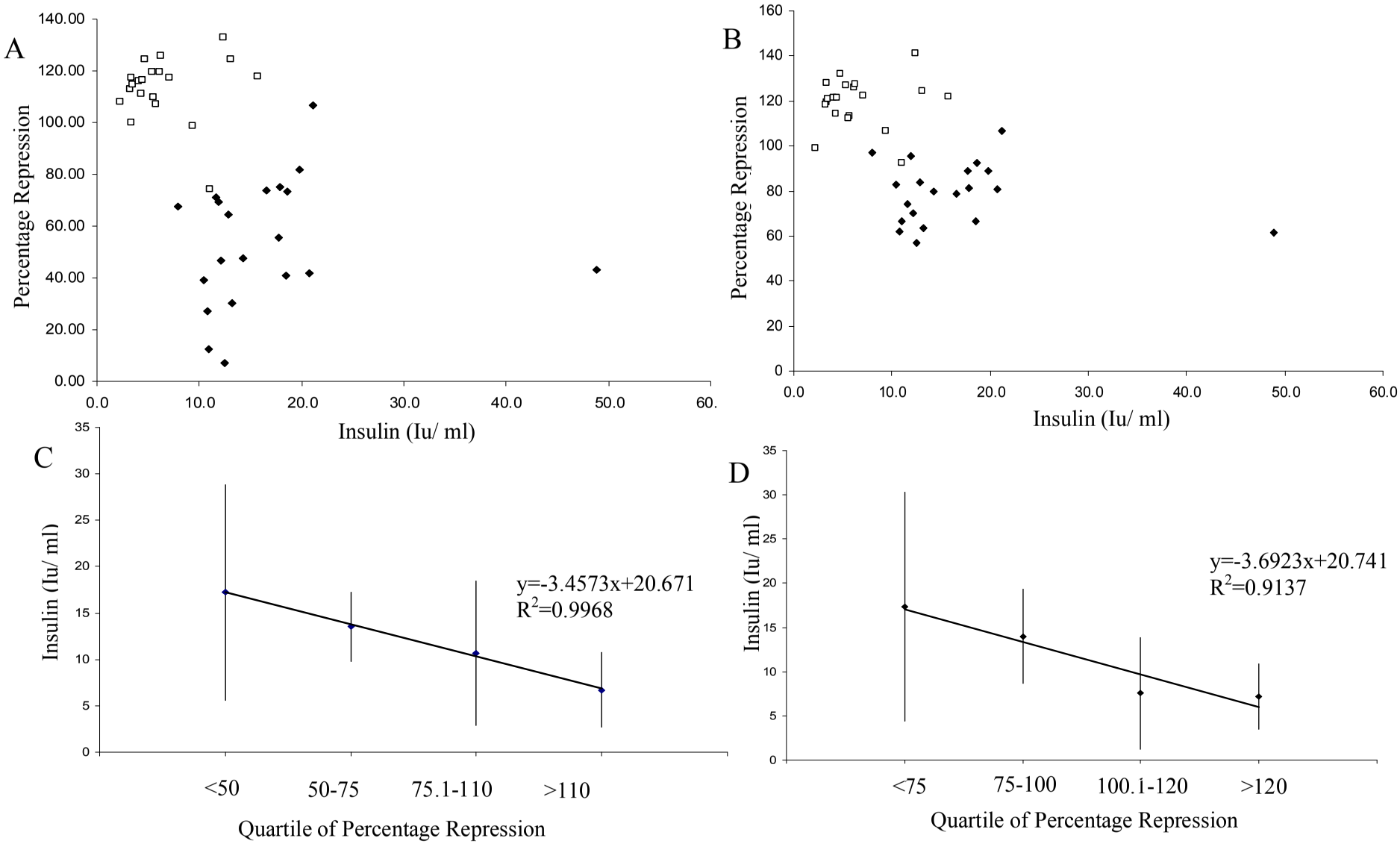


Figure 3.9 Scatter plot of Insulin levels and percentage repression of PEPCK by (A) 0.1 nM insulin, (B) 0.5 nM Insulin. All experimental subjects shown. Hollow squares= controls black diamonds = cases. (C) Quartile of percentage repression by 0.1 nM insulin against mean insulin level (D) Quartile of percentage repression by 0.5 nM insulin against mean insulin level Data shown as Mean +/- SD

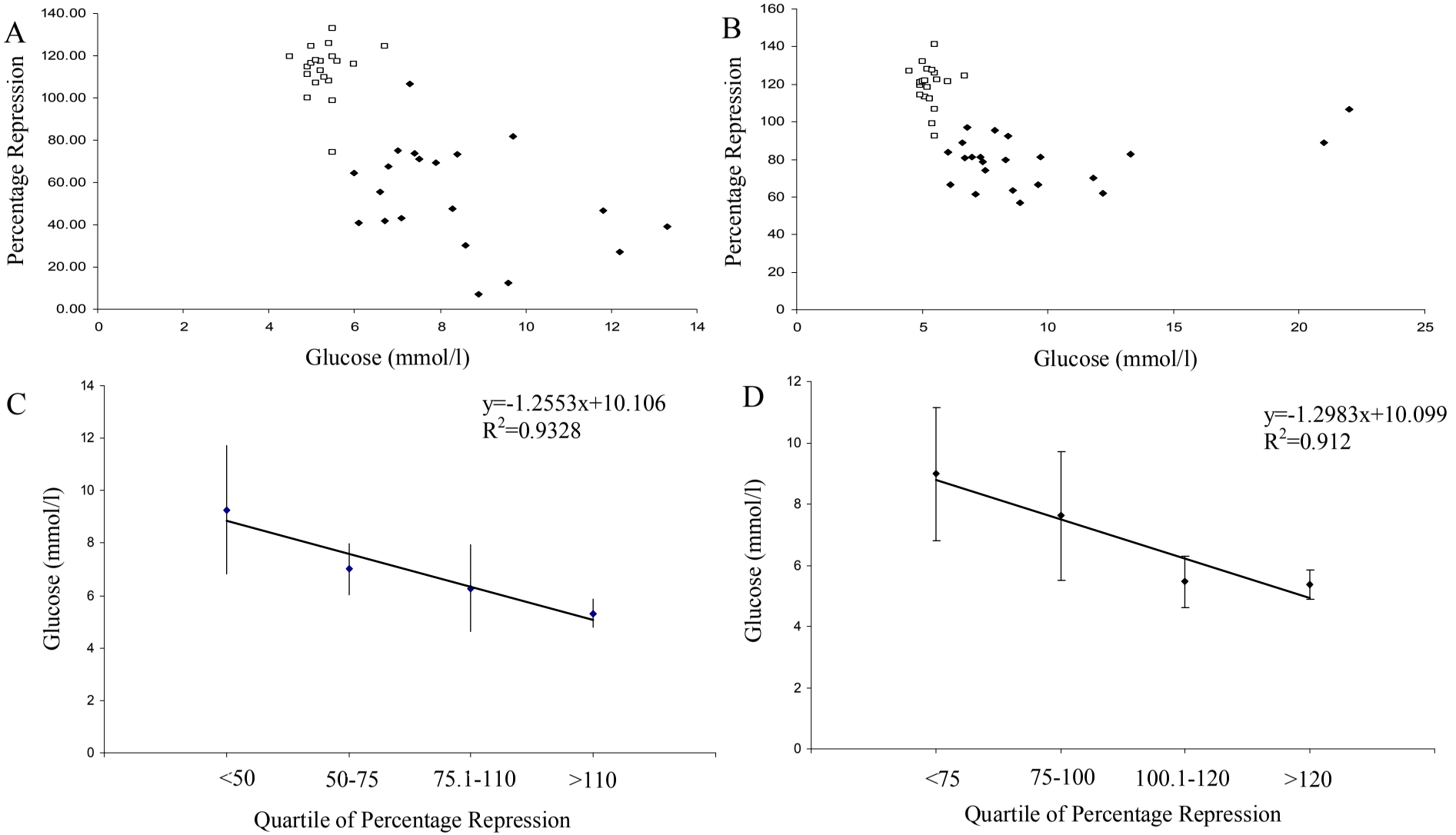


Figure 3.10 Scatter plot of fasting glucose levels and percentage repression of PEPCK by (A) 0.1 nM insulin, (B) 0.5 nM Insulin. All experimental subjects shown. Hollow squares= controls black diamonds = (cases) Quartile of percentage repression by 0.1 nM insulin against mean glucose level (D) Quartile of percentage repression by 0.5 nM insulin against mean glucose level Data shown as Mean +/- SD

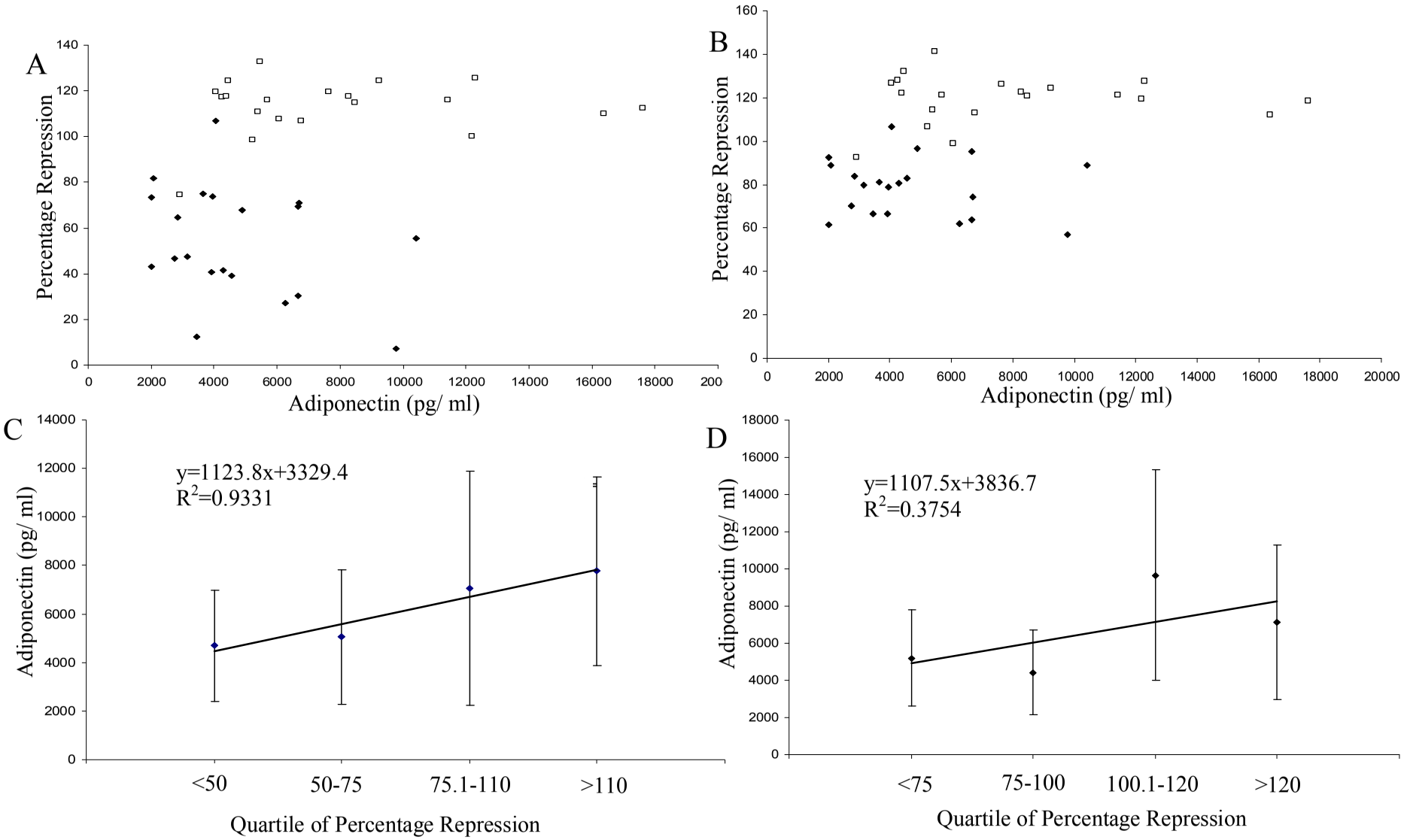


Figure 3.11 Scatter plot of adiponectin levels and percentage repression of PEPCK by (A) 0.1 nM insulin, (B) 0.5 nM Insulin. All experimental subjects shown. Hollow squares= controls black diamonds = cases (C) Quartile of percentage repression by 0.1 nM insulin against mean adiponectin level (D) Quartile of percentage repression by 0.5 nM insulin against mean adiponectin level Data shown as Mean +/- SD

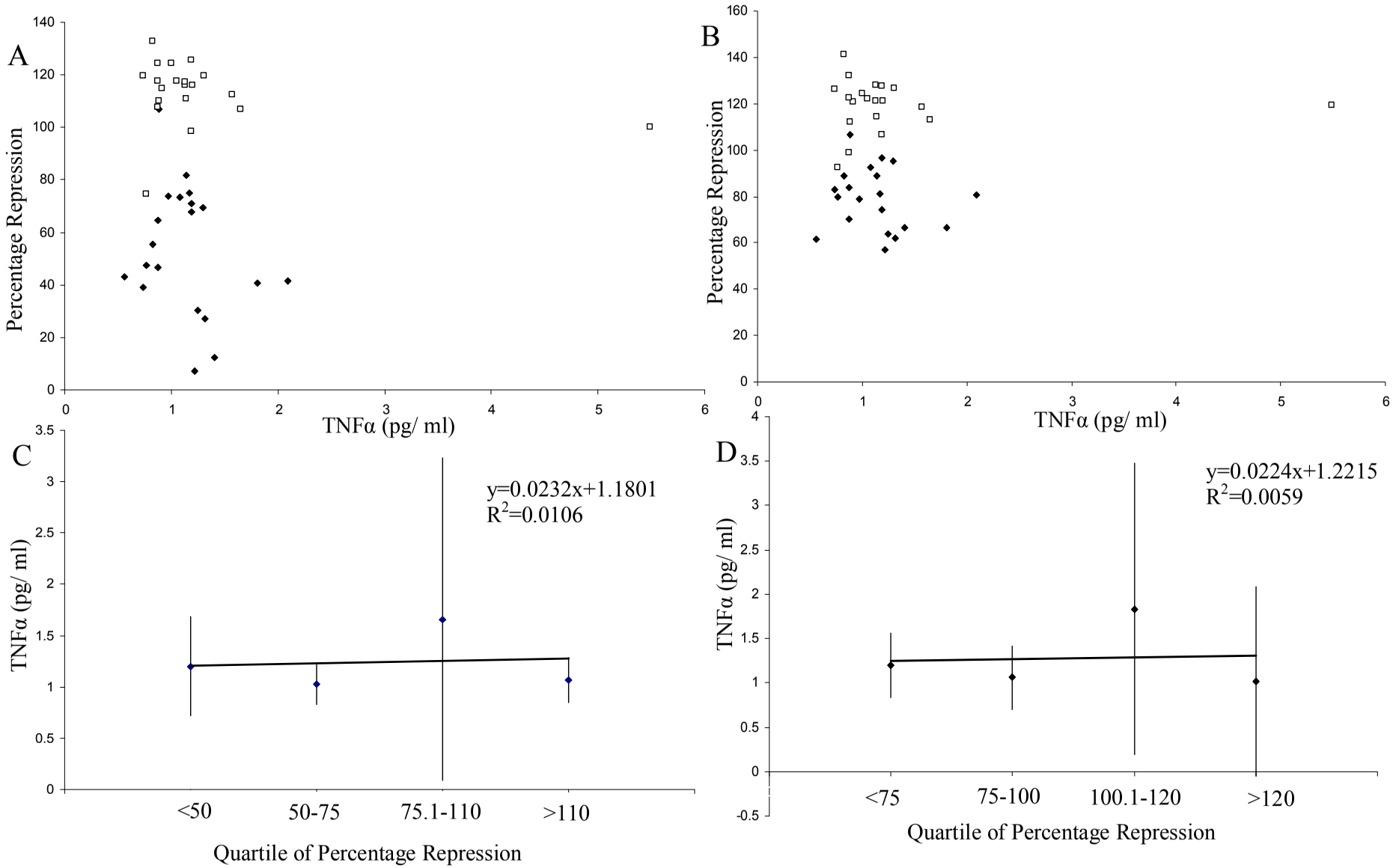


Figure **3.12** Scatter plot of TNFα levels and percentage repression of PEPCK by (A) 0.1 nM insulin, (B) 0.5 nM Insulin. All experimental subjects shown. Hollow squares= controls black diamonds = cases (C) Quartile of percentage repression by 0.1 nM insulin against mean TNFα level (D) Quartile of percentage repression by 0.5 nM insulin against mean TNFα level Data shown as Mean +/- SD

response to 0.1 and 0.5 nM insulin Figure 3.9 C and D).

Despite a significantly higher glucose level in the cases there was no linear relationship between fasting glucose level and the effect of the sera on the response of H4IIe cells to 0.1 or 0.5 nM insulin (Figure 3.10 A and B). Yet there is a strong correlation between fasting glucose levels and the effect of sera on the insulin sensitivity of the cells when the latter is grouped as quartiles (r^2 value of 0.93 and 0.91 respectively for 0.1 and 0.5 nM insulin Figure 3.10 C and D).

There is no linear relationship between adiponectin level and the response of H4IIe cells to 0.1 or 0.5 nM insulin (Figure 3.11 A and B). However, there is a correlation between plasma adiponectin and response of the cell model to insulin when the latter is grouped in quartiles, but this is only evident at 0.1 nM insulin (r^2 value of 0.93, Figure 3.11 C). There is no correlation when using data from cells exposed to 0.5 nM insulin (r^2 value of 0.13 Figure 3.11 D). Scatter plots show no relationship between plasma TNFα levels and the ability of the sera to alter insulin sensitivity of the H4IIe cells (Figure 3.12 A to D).

One of the potential aims of developing the cell based model was to determine whether it could be used in the future to identify individuals with pre-diabetes, at risk of progressing to full blown diabetes. As a first step to investigating this potential I attempted to define threshold values that would clearly dissociate cases from controls in the current study groups. I defined sera as insulin resistant if they altered the hormonal response of the H4IIe cells so that there was < 90% PEPCK repression by 0.1 nM insulin or <100% repression by 0.5 nM insulin (Table 3.3). To define the status of other biomarkers, the mid point between the means was used as a cut off. Any value nearer to

the mean of the cases was defined as being in the case group for this factor and vice versa. Data was analysed using 2 x 2 contingency tables looking at case/control status versus each factor. Sensitivity and specificity are statistical measures of a binary classification. Sensitivity is defined as the proportion of positives identified as such (the percentage of cases identified as insulin resistant by the cell model) and specificity measures the proportion of correctly identified (the percentage of controls identified as insulin sensitive by the cell model). This showed that there is a 95% sensitivity and specificity of the cell model predicting case-control status with 0.1 nM insulin (Table 3.3). This reduced to 95% sensitivity and 90% specificity when 0.5 nM insulin is used (Table 3.3). This is much better than any other measured serum biomarker, none of which reach a sensitivity of >80% (Table 3.3).

Adiponectin is particularly disappointing with a specificity of only 60% and a sensitivity of 70% (Table 3.3). It is not surprising that BMI has a 100% sensitivity and specificity, as this was one of the inclusion criteria by which controls and cases were defined (Table 3.3). Waist circumference is also 100% sensitive and specific, but this is intimately associated with obesity and as such would be expected to predict case and control status (Table 3.3).

In conclusion, it is clear that sera from obese diabetics has a significant effect on the insulin sensitivity of cells when compared to lean controls. However, despite measuring many clinical and biochemical parameters I could not identify a unique serum factor that correlated closely enough with the change in response of the cells to insulin to suggest it was responsible for the generation of insulin resistance.

	Midpoint of Means	Sensitivity (%)	Specificity (%)
Cell Model 0.1 nM Insulin	N/A	95	95
Cell Model 0.5 nM Insulin	N/A	95	90
BMI	29.2 kg/m^2	100	100
Waist Circumference	103.1 cm	100	100
Weight	92.1kg	95	95
HOMA-IR	1.45	95	80
Leptin	7087.372 pg/ml	95	75
Insulin	10.4 IU/L	90	80
HBA1C	6.02%	80	100
Glucose	6.84 mmol/l	75	100
Adiponectin	5619.23 pg/ml	70	60
CRP	1439	65	75
TNFα	1.11 pg/ml	45	50

Table 3.3 Sensitivity and specificity of different methods of predicting case and control status. The cell model used a cut off of <90% repression with 0.1 nM insulin and <100% with 0.5nM insulin to define the presence of insulin resistance. Other factors were assessed by using the mid point between means to define groups.

3.3. Discussion

There were three major reasons for trying to develop a cell based model of human insulin resistance;

To be used as a medium throughput screen to identify insulin sensitising agents,

To help identify factors that could promote insulin resistance and investigate the molecular mechanism (s) involved, and

To generate preliminary data that a cell based screen could help classify patients with diabetes by their molecular problem and thus guide choice of therapy.

The first step for all three goals was to extend previous work in the lab that generated insulin resistance in cells by culture in serum from obese rats. I aimed to demonstrate that similar effects could be obtained using serum from human volunteers. In this chapter I have described the recruitment and characterisation of two cohorts of volunteers both non-diabetic and diabetic. I confirmed a decrease in insulin sensitivity in H4IIe cells cultured in serum from insulin resistant individuals in comparison to cells cultured in control human serum. This clearly demonstrates that the model is applicable to the study of the human condition, and it is worthwhile developing the model to achieve the goals listed above.

3.3.1. Advantages of the cell model

There is a wealth of literature implicating many different factors associated with obesity in the development of insulin resistance. Hyperglycaemia (Oku et al., 2001) and hyperinsulinaemia (Rizza et al., 1985) are in themselves proposed to induce insulin resistance. It is as yet unknown whether this is the primary event in the human that leads to the development of insulin resistance, or whether it is a consequence of the disease progression. It is also known that high levels of serum triglycerides can promote both

hepatic and muscle insulin resistance (Prada et al., 2005). Unfortunately, triglycerides were excluded from my statistical modelling due to the confounding factor of HMG CoA reductase inhibitor usage even though the cases retained significantly higher levels triglycerides. Free fatty acids have also been shown to induce insulin resistance by negatively regulating insulin signalling (Seppala-Lindroos et al., 2002), and these are increased in diabetes associated with obesity. Other markers of obesity such as high TNFα (Uysal et al., 1997, Plomgaard et al., 2005), low adiponectin (Hotta et al., 2001), high leptin (Benomar et al., 2005) and high IL-6 (Kroder et al., 1996, Mooney et al., 2001) are also proposed to reduce insulin action by negatively regulating the insulin signalling pathway.

This suggests either that there are multiple different ways obesity can lead to insulin resistance or that each molecule contributes to only a part of the disease process. In the first scenario insulin resistance as observed in the clinic would actually be due to many different molecular diseases and potentially would require different therapeutic interventions. In the latter scenario a therapeutic effective against only one of the obesity-related factors involved would have limited clinical efficacy. Therefore a better understanding of the molecular development of diabetes associated with obesity is clearly of clinical importance. Serum has been stored for future metabolomic analysis to describe the differences between cases and controls in their metabolic profile.

We decided to take an unbiased approach to the development of a cellular model of insulin resistance. Culturing cells in serum from obese patients with diabetes would hopefully generate a form of insulin resistance mechanistically related to that seen in the disease group of interest. It now also allows us to try to identify the actual serum

component responsible, and establish whether it is a single factor, and whether it is the same factor (s) in every patient.

We have used the PEPCK gene promoter as a surrogate of insulin action. PEPCK is the endpoint of the major insulin signalling pathway downstream of the IRS-PI 3-K pathway. This signalling pathway contributes to most of the major metabolic actions of insulin, hence factors which improve sensitivity to insulin in this screen are likely to improve many actions of insulin. The loss of insulin sensitivity is a shift in, rather than complete abrogation of, insulin action, and higher doses of insulin still completely repress PEPCK even in the most insulin resistant cases. This is more in keeping with the physiological disease state where obesity does not cause complete loss of insulin action and does not promote disease overnight.

Another major use of the model would be a drug screen for insulin sensitisers and insulin mimetics. This has the advantage that any compounds that are effective in the model should be effective within the target population that donated the serum for the screen. It will also allow relatively rapid assessment of drug efficacy prior to proceeding to animal testing, thereby reducing the number and cost of research animal usage.

3.3.2. Disadvantages of the cell model investigated here

There is significant variation in insulin sensitivity in the cases as measured by the cell model. There is less variability in the controls which virtually all show more than 90 and 100% repression of PEPCK with 0.1 and 0.5 nM insulin. However, it has been known for some time that there is large variation in insulin sensitivity present in the human population, even in healthy cohorts measured by the sensitive hyperinsulinaemic euglycaemic clamp method (Petrie et al., 1996). This could reflect the range of potential mediators of insulin sensitivity present *in vivo* and in serum, and suggests that it is

unlikely that a single biomarker exists that will diagnose insulin sensitivity deficits related to disease progression. Consistent with this, it was not possible to identify a single factor responsible for the generation of insulin resistance in the cell model using regression or linear association analysis. Interestingly, this variability even occurs in inbred rodent strains where animals placed on the same high fat diet develop different levels of obesity, insulin resistance and hyperglycaemia (Chapter 1.13.1.3). It is unlikely that the subtle changes in insulin sensitivity seen in this model will be apparent as large perturbations of the known signalling pathways if analysed by Western blotting. Proteomic comparison of these cell cohorts may allow a more refined analysis of known pathways to identify where any molecular deficits lie. The serum could also be subjected to proteomic, metabolomic and transcriptomic analysis to identify novel markers of insulin resistance. Ideally, this model could also be extended to other tissues, such as adipose, β-cells and skeletal muscle. For this to be a robust process, an accurate readout, such as PEPCK in hepatoma cells, would be required.

The serum used in our study was obtained from peripheral blood. Blood from the portal circulation, to which the liver is usually exposed, has a different makeup (e.g. it will contain higher levels of glucose, lipids, amino acids, insulin, gut incretins and decreased glucagon concentration) and how this affects insulin sensitivity may be completely different from the effects of peripheral serum. However, obtaining human portal serum is not possible in the quantities required to generate the model.

There is clearly a difference between insulin sensitive lean individuals and obese diabetics. However, insulin resistance is a continuum rather than a dichotomy. It would be useful to expand the groups to include obese non-diabetics and lean diabetics to assess whether the model is truly reflecting an aspect of obesity or a factor subsequent

to the development of type 2 diabetes. Indeed, it would be worthwhile performing a prospective study where we follow obese non-diabetics and establish if the model can identify those that go on to develop type 2 diabetes.

The confounding use of HMGCoA reductase inhibitors may also have had an effect on the insulin resistance causing ability of serum from the cases. A recent meta-analysis of large scale clinical trials has shown that there may be an increase in the risk of developing type 2 diabetes in those taking statins (Rajpathak et al., 2009). However, the mechanism for this is not yet fully understood, and most theories appear to centre on their effects on β-cells and adipose tissue rather than liver (Ishikawa et al., 2006a, Takaguri et al., 2008, Ishikawa et al., 2006b, Takagi et al., 2008, Koh et al., 2009).

To use the cell model in its current form as a drug screen would require significant development. An ideal compound screen is rapid, automated, cheap and robust. The multi-step nature of RNA extraction and Taqman assay make the analysis relatively slow and expensive for a large scale format. Ideally, a cell based screen would use reporter cell lines where the production of an easily quantifiable marker under the control of the PEPCK gene promoter could be analysed in a 96 well format. As well as this, it would be of interest to look at other gluconeogenic and insulin responsive genes, for example G6Pase and IGFBP-1 (Chapter 1.9.3 and 1.9.3), to assess whether any loss of insulin sensitivity observed measuring PEPCK was extended to other insulin responsive genes. The development of such reporter cell lines forms the basis of Chapter 4.

It would be of interest to follow the control group to determine whether any progress to diabetes. Only a couple of the control sera affected the insulin response of the H4IIe

cells within the range of response seen with the case sera. For example, cells grown in sera from control 10 had a relatively poor PEPCK suppression by insulin at both 0.1 and 0.5 nM (Figures 3.3 and 3.4). Interestingly, this volunteer had the lowest level of adiponectin within the control group, and is actually lower than many of the cases. Control 10 also has a leptin level approximately 3 times higher than the mean of the control group (6376.89 compared to 1198.39), although not the highest within the group.

Similarly, there are case sera which produce cell responses more similar to control sera. The best example of this is case number 33 (Figures 3.5 and 3.6). Basal PEPCK mRNA, and fold induction of PEPCK, in cells grown in case 33 are similar to the mean of the control group (Figures 3.3 and 3.4), and the percentage repression by 0.1 and 0.5 nM insulin is more in line with the effect of control sera rather than case sera (Figure 3.5 and 3.6). There is no outstanding feature of the biochemistry of this sample that would explain the lack of development of insulin resistance. Volunteer 33 has a BMI of 34, hyperleptinaemia (14717.22 pg/ml), hyperinsulinaemia (21.2 iU/ml), high CRP (>5000) and is clearly insulin resistant (HOMA-IR of 2.9), all parameters higher than the mean for the cases. Likewise, adiponectin levels (4039 pg/ml) are lower than the mean for cases. Interestingly, fasting glucose and HbA1c are both lower than the mean for cases, so it is possible that good glucose control is having an effect on the sera component involved in generation of insulin resistance in the cells in culture.

3.4. Conclusions

I have developed a humanised model of insulin resistance which can be used to investigate the link between obesity and hepatic insulin resistance. Unfortunately, this initial study has not identified individual serum factors that could be responsible for the development of insulin resistance in the cells exposed to diabesity sera. It is likely that

there is a complex interplay between many of the factors involved. Thus, assessing the effects of several factors, individually and in combination, at the concentrations seen in the diabesity serum, may be a useful approach. This would be made technically possible by the development of reporter cell lines.

Chapter 4. Development of Reporter Cell Systems as Screens for Insulin Sensitisers

4.1. Introduction

Drug discovery is a long process during which compounds are developed through several phases from identification to clinical use. Initial identification of novel small molecules is usually achieved by high throughput screening using assays for therapeutic targets (e.g. enzymes, protein-protein interactions, receptor agonists/antagonists etc). Lead compounds from initial screens are often made more 'druggable' by intelligent design (e.g. reduce molecular weight by identifying key motifs in structure or change hydrophobicity to improve pharmacokinetics). These optimised leads then undergo larger scale synthesis and selectivity characterisation. Ultimately the novel compounds need to be screened for efficacy and safety, which involves animal studies prior to clinical trials. The development costs and time start to escalate exponentially at the *in vivo* study stage therefore the more *in vitro* or cell based screens that can be applied prior to this stage the better. Clearly, establishing meaningful and informative cell based secondary screens is a vital part of drug development. The ideal cell based model to identify agents that would improve insulin sensitivity in a patient population would be a humanised insulin resistant system. It should be relatively inexpensive, be of medium throughput (96 well automated formats with procedure times of less than 24h) and be fully quantitative.

There are a number of cell based systems available for screening the effects of compounds on the action of insulin. It is possible to assess the effects a compound has on the insulin signalling pathway, such as reduction or enhancement of the phosphorylation of PKB, measured by Western blotting or Mass Spectrometry. This is a relatively slow process, but can be useful in hepatic cell models where glucose uptake is a less useful tool. However, this marker of insulin sensitivity tends to have a very narrow range of insulin response, that is, small changes around the EC50 tend to have

large effects making the variability or noise of the assay problematic. Glucose uptake assays in muscle (Bilan et al., 1992) or adipose tissue (Frost and Lane, 1985) can also be utilised in efforts to identify compounds which modulate this insulin action. Although this is a physiological output, it is of less use in assessing changes in hepatic insulin sensitivity as glucose uptake in this tissue occurs through passive diffusion and is only indirectly related to insulin sensitivity of the cells. Also, this technique is low throughput, requires the use of radioactive isotopes and is costly. Finally, GLUT4 translocation assays can be used as a surrogate marker of glucose uptake. Traditionally, the use of this technique required semi-quantitative imaging techniques. However, there have been recent developments to automate and quantify this process (Liu et al., 2009). The major criticisms of all of the above models is that compounds are often used to improve sensitivity in cells with physiological insulin sensitivity, or that resistance is induced by methods biased toward a specific factor of particular interest to the researcher, such as culturing in high levels of palmitate. The importance of developing an insulin resistant cell model lies in the fact that the effects of any compounds screened should translate to the clinic (human insulin resistance). By culturing H4IIe cells in human serum and using PEPCK mRNA as a readout of insulin sensitivity, an unbiased model of insulin resistance has been developed, and it may be possible to use this model to identify clinically effective insulin sensitisers.

Extraction of mRNA, cDNA manufacture and Taqman analysis as described in the previous chapter does not fulfil the criteria of expediency and frugality, and so a different strategy was needed. An alternative approach is to generate stable cell lines incorporating recombinant genes which express easily quantifiable products under the control of gene promoters of interest (e.g. the PEPCK gene promoter).

Reporter assays using luciferase are most widely used due to their ease and rapidity of assay, high sensitivity, low cost and low endogenous activity. This protein is also stable and relatively non-toxic even at high levels in eukaryotic cells. Firefly luciferase is an enzyme that catalyses the oxidation step of luciferin, a process which is ATP dependent (Branchini et al., 1998). The reaction equation is

$$\text{Luciferin+Luciferase+ATP+}O_2 \longrightarrow \text{Oxyluciferin+Luciferase+AMP+Light+}CO_2$$

The luciferase enzyme activity (light emitted) can be used as a surrogate of gene promoter activity. I decided to investigate whether this system could be used to develop a cell based model of insulin resistance. By fusing the gene promoters of gluconeogenic proteins to the firefly luciferase cDNA and stably inserting it into the H4IIe cell genome, luciferase activity in the cells should be directly proportional to the activity of those promoters, allowing simple assessment of hormone action in these cells.

In this chapter I describe the manufacture and characterisation of reporter cell lines expressing luciferase in response to the transcriptional activity of the promoters of rat PEPCK and human PEPCK, G6Pase and IGFBP-1 genes. I go on to describe my attempt to develop a cell based model of insulin resistance using these reporter cells.

4.2. Results

4.2.1. Rat PEPCK gene promoter-luciferase reporter cells

4.2.1.1. Production of reporter cells with rat PEPCK gene promoter

The first stage of generating a reporter cell line was to clone a large section of the rat PEPCK promoter into the pGL4.17 vector from Promega (Figure 4.1 and 4.2, performed by Lisa Logie). Firstly, 2189 base pairs (bp) of the rat PEPCK promoter were cloned by PCR from rat genomic DNA using primers designed to include 68 bp 3' of the transcription start site (TSS) and 2121 bp 5' of the TSS, as shown (Table 4.1). The PCR product was purified by agarose gel electrophoresis and ligated into the TOPO 2.1 subcloning vector prior to full sequencing (Chapter 2.2.13). The vector pGL4.17 was linearised and the confirmed rat PEPCK gene promoter sequence isolated from TOPO 2.1 using SacI and BglII restriction enzymes. The PCR fragment was ligated into the cut pGL4.17 vector and positive clones propagated in competent *E.Coli*. The pGL4.17 containing the rat PEPCK plasmid was confirmed by in house sequencing (Appendix 2).

The PGL 4.17 rat PEPCK construct (Figure 4.2) was transfected into H4IIe rat hepatoma cells using the calcium phosphate precipitation method (Chapter 2.2.19). The cells were cultured in the presence of neomycin to select for transfected cells only as the pGL4.17 includes a neomycin resistance gene. Once colonies of resistant H4IIe cells were established, they were isolated and cultured separately. Eight such colonies were chosen for characterisation (LLRP1-8 = Lisa Logie Rat PEPCK and denotes who undertook the transfection and selection of colonies containing the reporter cassette). Cells from all colonies were frozen at -80 °C and transferred to liquid nitrogen for long term storage (Chapter 2.2.2.3).

Primer Name	Primer Sequence (5' to 3')	From-to	PCR Product Size
Human G6PAse Forward (hG6Ps5prSalI)	GTCGACCCTTTGAGAATCCACGGTGTC	-2785 to +85	2870
Human G6PAse Reverse (hG6Ps3prHind)	AAGCTTAGGTGCCAAGGAAATGAGG		
Human IGFBP-1 Forward (hBP15prSalI)	GTCGACGTTCCCAGAAATGGTGTGTG	-2957 to +229	3186
Human IGFBP-1 Reverse (hBP13prHind)	AAGCTTAAACTCTGGGCAAGTGATGC		
Human PEPCK Forward (humPCKf)	ATTCCTTTCTCGACCCTCGTC	-2991 to +105	3096
Human PEPCK Reverse (humPCKr)	ATCTCGAAGGGAGATCCACAG		
Rat PEPCK Forward (PCRPCKfor)	GTTACGGTACCGTACGAGCGCTGAACATCACAC	-2121 to +68	2189
Rat PEPCK Reverse (PCRPCKrev)	GCTTACGCTCGAGCTCCTTTTGGAAGGTCTGGA		

Table 4.1 List of primers used to generate PCR products from genomic DNA. PCR products are the gene promoters of the specified genes for cloning into pGL 4.17

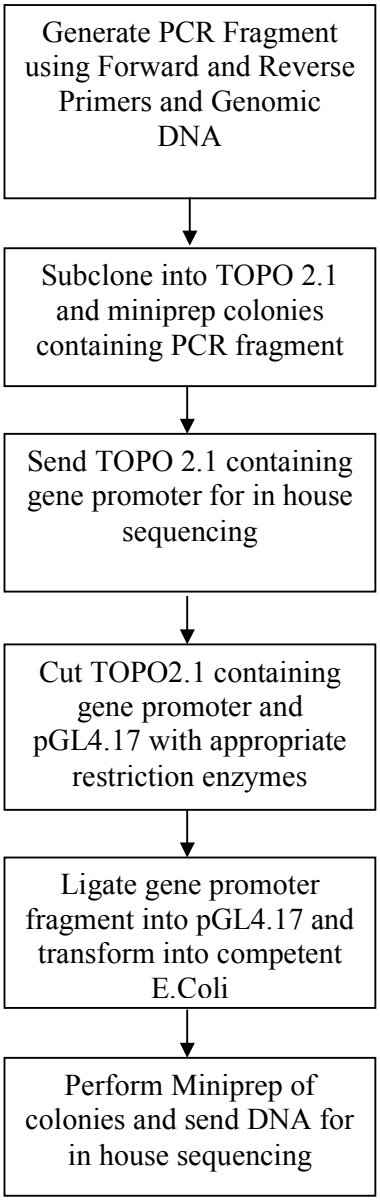


Figure 4.1 Cloning strategy for creating pGL4.17 containing the gene promoters driving luciferase production

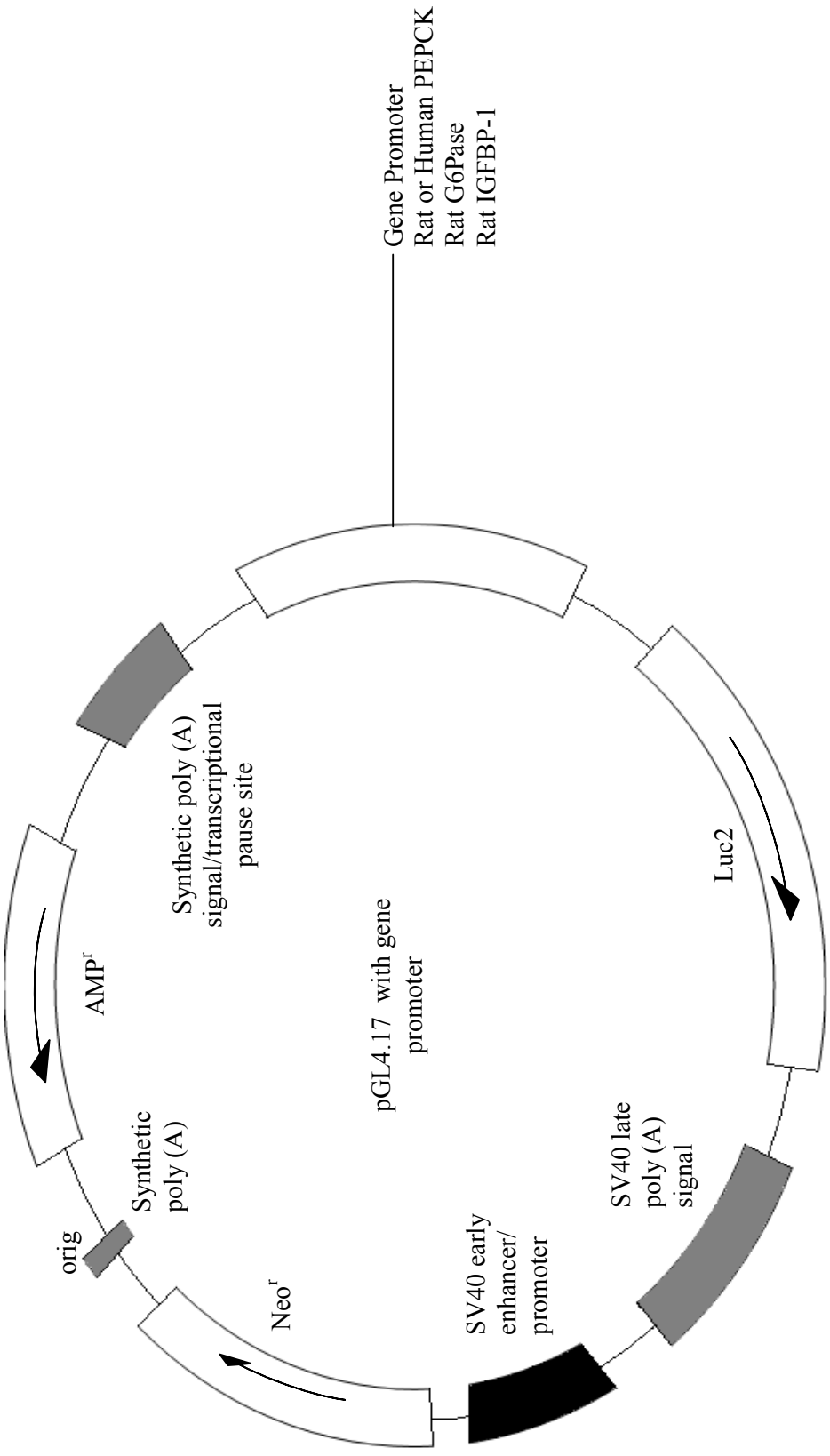


Figure 4.2 Vector Map of pGL4.17 containing the an insulin responsive gene promoter. Luc2 = luciferase sequence, AMP^r = Ampicillin resistance cassette, Neo^r = Neomycin resistance cassette, orig = origin of replication

4.2.1.2. Characterisation of LLRP Cells

In an attempt to assess whether the stably inserted recombinant rat PEPCK promoter luciferase reporter was under normal hormonal control, all 8 colonies of LLRP cells were plated and allowed to reach 60% confluence. The cells were fasted for 3 hours prior to treatment for 16 hours with serum free media or dexamethasone (500 nM) and the second messenger cAMP (100 μM 8-CPT cAMP) in the presence or absence of increasing concentrations of insulin. The cells were lysed and assayed for luciferase activity (Chapter 2.2.20.1). All 8 colonies were found to be stimulated by dexamethasone and cAMP and in all cases induction was dominantly repressed by insulin (Figure 4.3).

However absolute luciferase expression was variable between colonies (Figure 4.3), suggesting this was influenced by position and/or number of gene insertion. However the fold change by the addition of dexamethasone and cAMP was similar ranging from 10 times for colony 5 to 15 times for colony 6 (Figure 4.4 A). The percentage reduction from maximal stimulation by the addition of 10 nM insulin ranged from 50% for colony 4 to 68% for colony 7 (Figure 4.4 B). PEPCK gene transcription is regulated within minutes of cellular exposure to hormones, and changes in mRNA are apparent between 1 and 3 hrs after exposure. Therefore to investigate optimal exposure time for assessment of luciferase activity the regulation of LLRP-3, -7 and -8 lines was further investigated by varying the length of time exposed to hormones. Cells were fasted for 3 hours and then treated for either 3 or 16 hours with or without dexamethasone and cAMP in the presence or absence of increasing doses of insulin. At 3 hours stimulation of the cells with dexamethasone and cAMP, the fold change in luciferase activity was only 2.2, 2.3 and 2.1 respectively (Figure 4.5). This was much weaker than the stimulation at 16 hours (7.8, 10.6 and 11.2 fold for LLRP 3, 7 and 8 respectively)

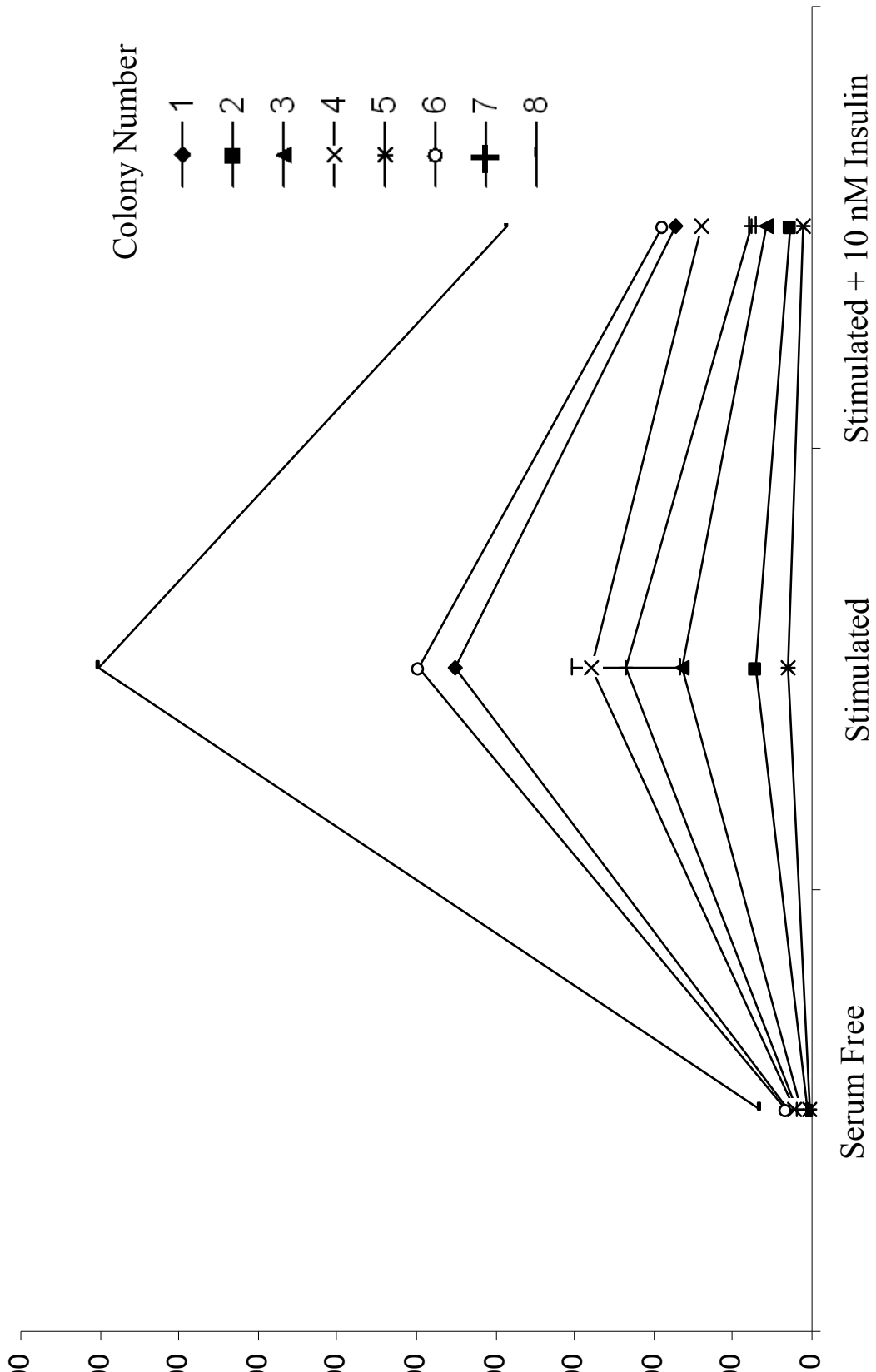


Figure 4.3 Colony testing of H4IIe cells stably transfected with pGL4.17 rat PEPCK. Cells were starved for 3 hours then treated for 16 hours with or without dexamethasone and cyclic AMP in the presence or absence of insulin before lysis and assay of luciferase activity. All tests performed in duplicate and shown as mean relative light units (n=2-4). Error bars shown only for Colony 7 to shoe significant stimulation and repression

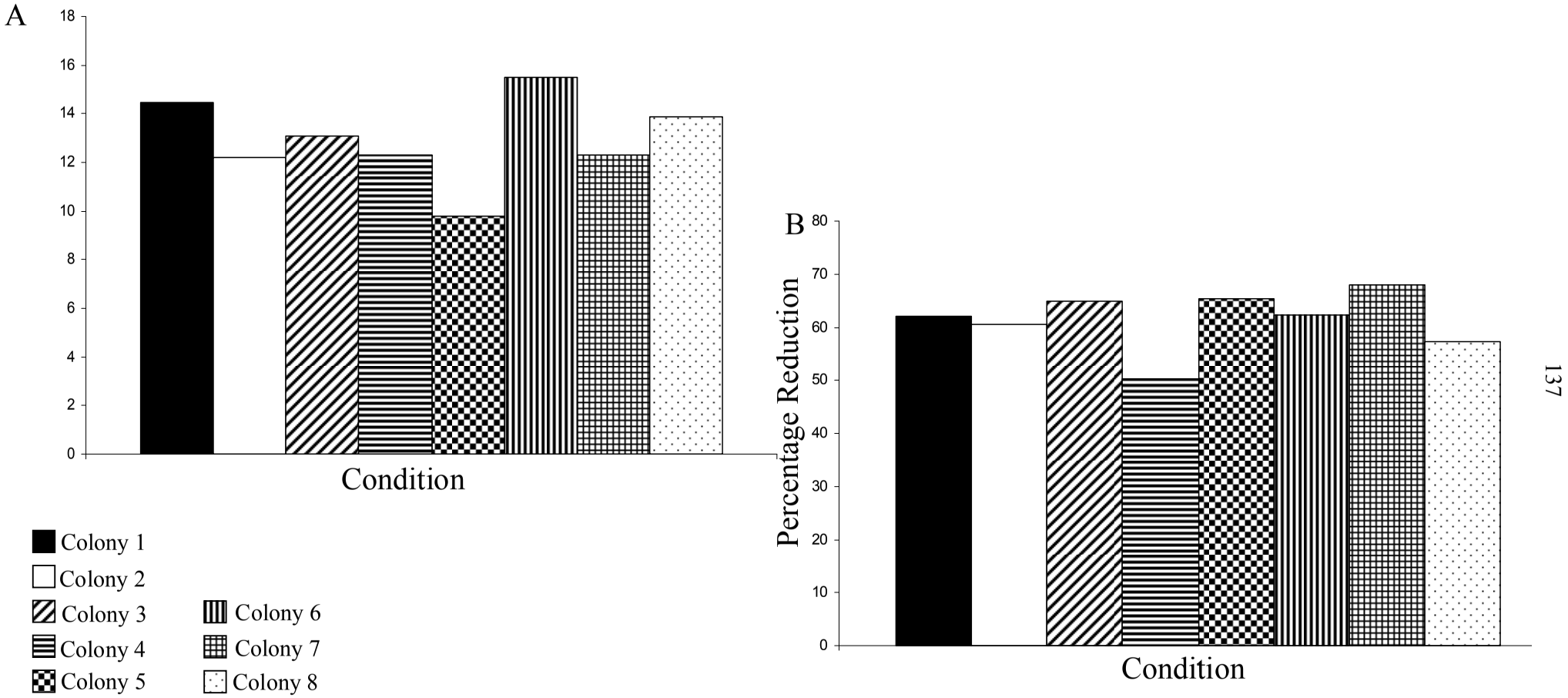


Figure 4.4 Testing of H4IIe cells stably transfected with pGL4.17 Rat PEPCK. (A) Fold stimulation of relative light units from basal levels by the addition of dexamethasone and cyclic AMP. (B) Percentage repression of relative light units from maximal stimulation with dexamethasone and cyclic AMP by the addition of 10 nM Insulin (n=2-4 for each clone)..

(Figure 4.5). The percentage repression by 10 nM insulin at 3 hours was 63.1, 64.6 and 33.1% in LRRP 3, 7 and 8 respectively (Figure 4.5). At 16 hours a similar response to insulin was obtained in at least 2 of the lines (68.7, 64.3 and 70.8% repression respectively) (Figure 4.5). As the response of the 3 lines was quite similar at the longer incubation times it was decided to focus on LLRP7 for further characterisation of insulin signalling to the recombinant gene and in the development of an insulin resistant model. All further experiments involved hormone exposure for 16 hours unless stated.

The effect of higher doses of insulin (10 nM) on the repression of luciferase activity was not as great as one would expect i.e. complete repression of luciferase activity. Therefore, the effect of insulin alone on luciferase expression was assessed. LLRP7 cells were fasted for 3 hours prior to incubation with 0.1, 1 or 10 nM insulin for 16 hours before lysis and assay of luciferase activity. There was a 2-, 4.3- and 10.7-fold increase in luciferase expression by the addition of 0.1, 1 and 10 nM insulin respectively (Figure 4.6). This suggests there is an insulin enhancer within the reporter gene cassette that responds to higher concentrations of this hormone, thereby complicating our analysis of the PEPCK promoter. All further experiments investigating insulin action were performed at 1 nM insulin to optimise repression of luciferase and minimise induction of the enhancer.

Regulation of the endogenous PEPCK gene promoter by insulin involves the PI 3-K, PKB and GSK-3 signalling pathway (see Chapter 1.6 and 1.11 for details). In order to establish whether the recombinant gene in LLRP7 cells maintained the same signalling connections downstream of the insulin receptor the cells were fasted for 3 hours prior to 30 minutes pre-incubation the PI 3-K inhibitor PI-103 followed by stimulation with dexamethasone (500 nM) and the second

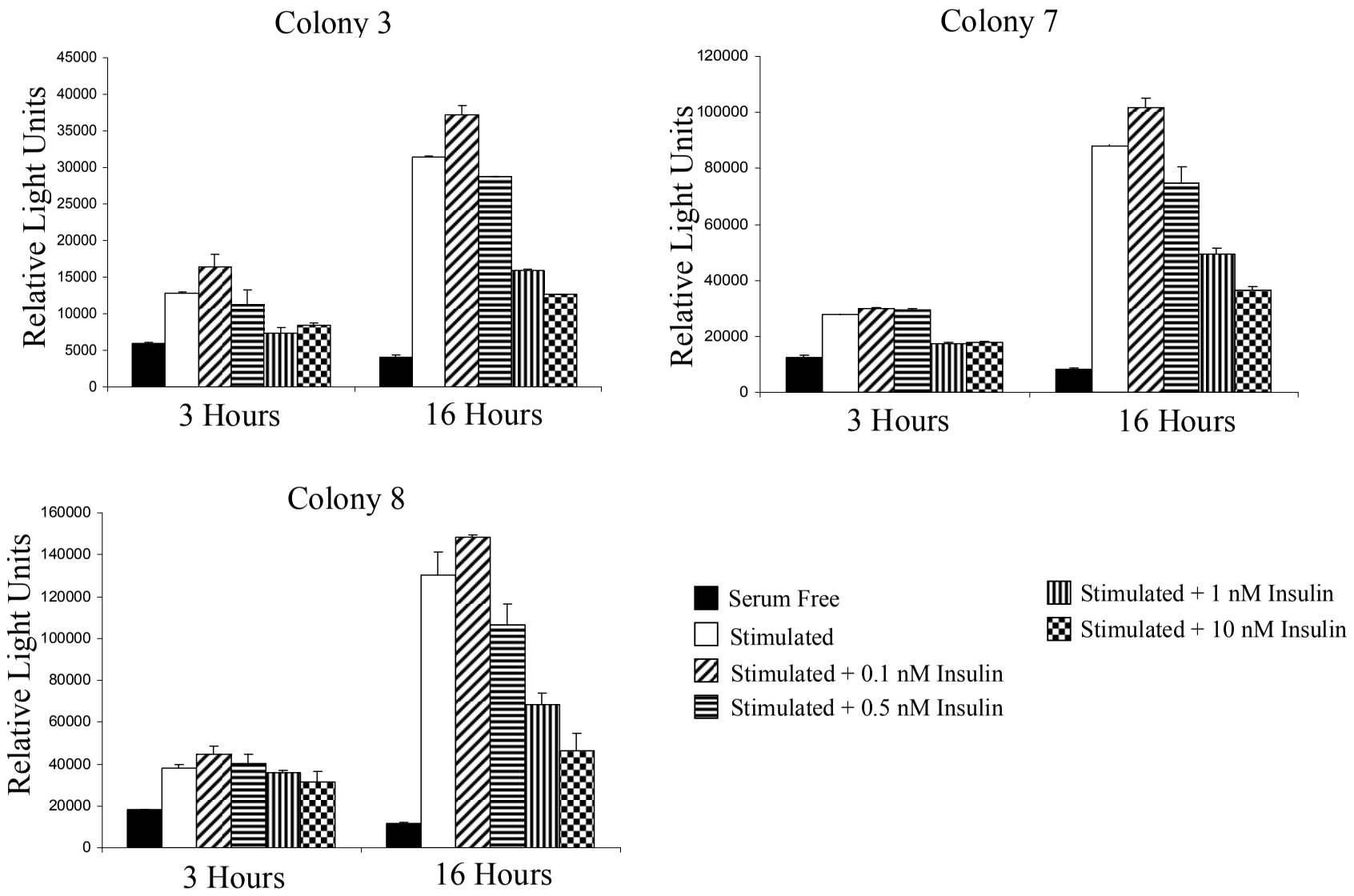


Figure 4.5 Colony testing of H4IIe cells stably transfected with pGL4.17 rat PEPCK. Cells were starved for 3 hours then treated for lengths of time as indicated with dexamethasone and cyclic AMP in the presence or absence of insulin before lysis and assay of luciferase activity. (n=2 for each clone at each timepoint)

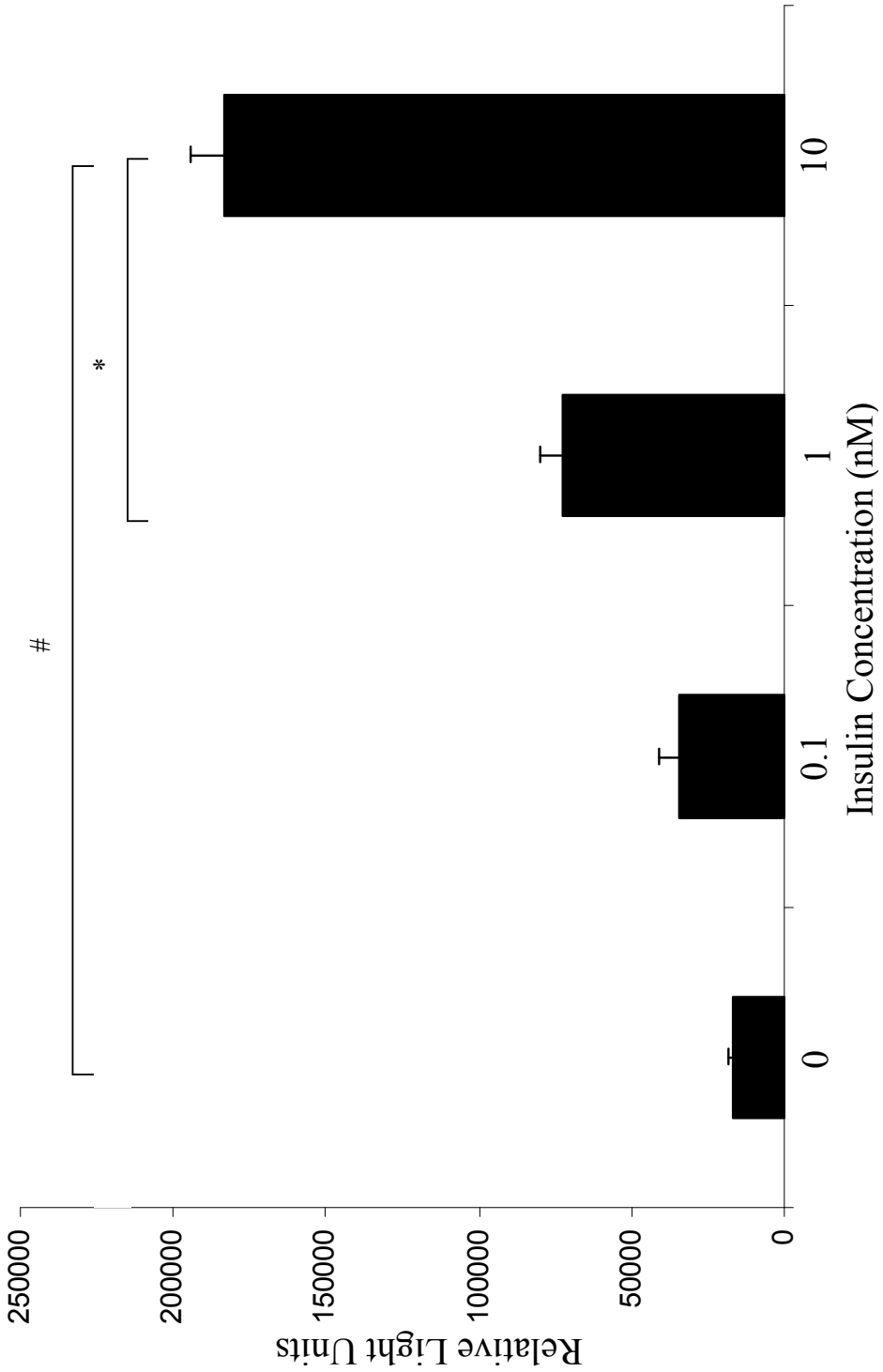


Figure 4.6 Effect of insulin on luciferase activity in LLRP7 cells. Cells were fasted for 3 hours prior to incubation with insulin as indicated before lysis and measurement of luciferase activity (n=2). P values, #=0.002, *=0.01

messenger cAMP (100μM 8-CPT cAMP) in the presence or absence of 1 nM insulin for 16 hours (Figure 4.7 A). The addition of PI-103 slightly increased the stimulatory effect of dexamethasone and cAMP (Figure 4.7 A). However, this PI 3-K inhibitor did not block the repressive effects of insulin on luciferase activity (Figure 4.7A). The half life of PI-103 is short, and it is likely that the incubation time for the reporter assay (16-17h) was too long for it to be effective over the whole period. Therefore, LLRP7 cells were exposed to a more stable PI 3-K inhibitor, LY294002, the GSK-3 inhibitor CT99021 or the PKB inhibitor Akti 1/2 (Figure 4.7 B). In this case inhibition of PI 3-K had a much greater effect on the stimulatory effect of dexamethasone and cAMP, and completely blocked any repression of luciferase activity by insulin, similar to its effects on PEPCK gene transcription (Figure 4.7 B). The inhibition of PKB blocked the insulin mediated suppression of luciferase activity consistent with the effects on the PEPCK gene. Surprisingly, the inhibition of GSK-3 prevented, rather than mimicked, insulin stimulated suppression of PEPCK activity. (Figure 4.7 B). In summary, LLRP7 cells are a stably transfected reporter cell line that expresses luciferase under the control of 2 kbp of the rat PEPCK gene promoter. The reporter, like the endogenous gene, is responsive to stimulation by dexamethasone and cAMP, and is dominantly repressed by the addition of insulin. This is most robust with 16 hours of hormone treatment. Insulin signalling connecting the receptor to the recombinant gene promoter appears similar to that reported for the endogenous gene promoter since loss of PI 3-kinase or PKB signalling prevents the insulin repression of the reporter. However, GSK-3 inhibition fails to repress the reporter production.

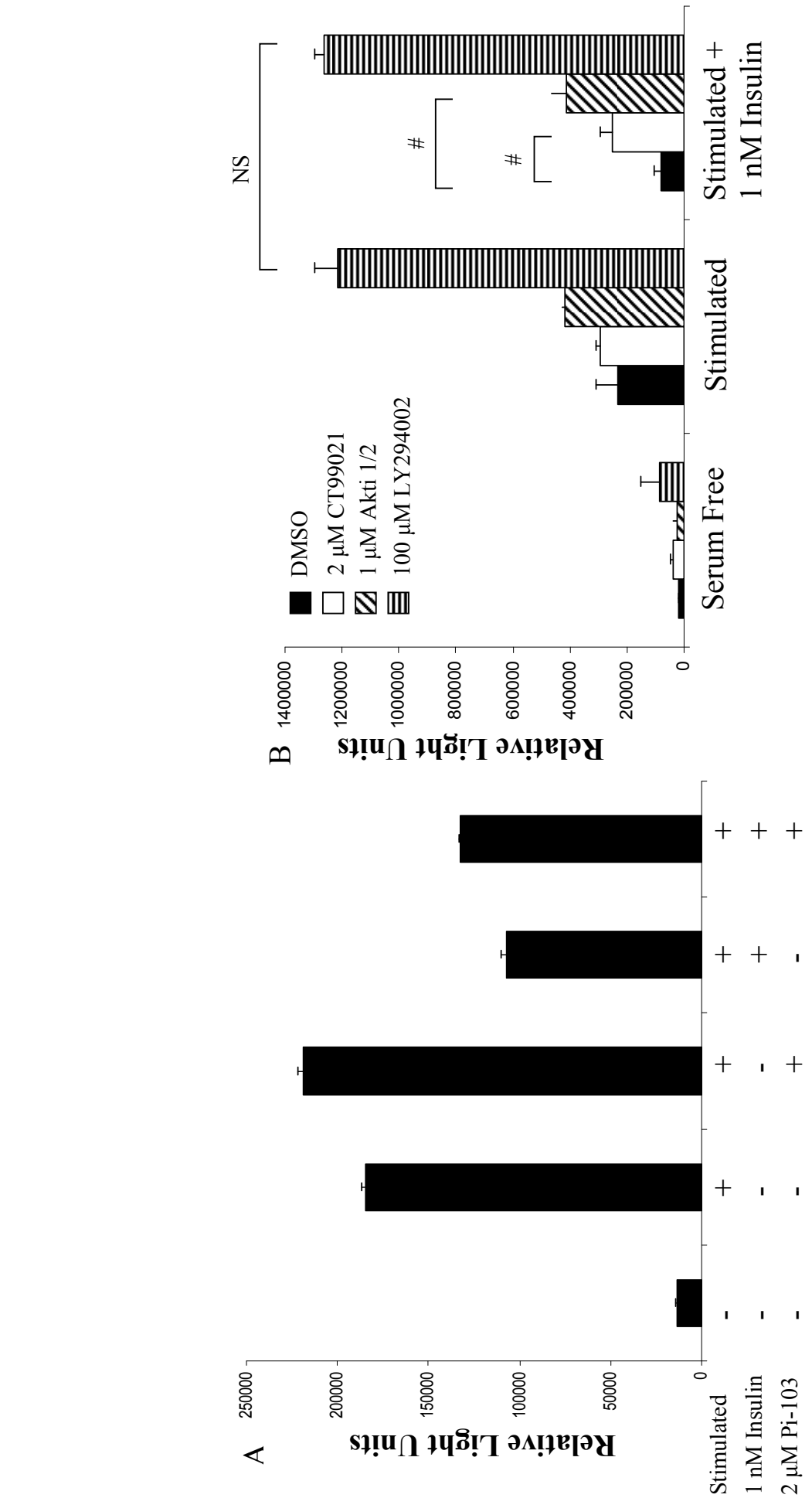


Figure 4.7 Effect of inhibition of GSK-3 by CT99021, PI 3-K by PI-103 and LY294002 and PKB by Akti 1/2 on H4IIe cells stably transfected with pGL4.17 rat PECK. LLRP7 cells were fasted for 3 hours before pre-treatment with indicated inhibitors for 30 minutes. Cells were then treated for 16 hours as indicated. (A) Effects of PI-103 (n=2). (B) Effects of LY294002, CT99021 and Akti 1/2. (n=2). P values, NS=nonsignificant, #=0.03

4.2.2. Human PEPCK gene promoter-luciferase reporter cells

4.2.2.1. Production of reporter cells with human PEPCK gene promoter

Firstly, 3096 bp of the human PEPCK promoter were cloned by PCR from human genomic DNA using primers designed to include 105 bp 3' from the TSS and 2991 bp 5' from the TSS, as shown (Table 4.1, Figure 4.1 and 4.2 performed by Lisa Logie)). The PCR product was purified by agarose gel electrophoresis and ligated into the TOPO 2.1 subcloning vector prior to full sequencing (Ch 2.2.13). The vector pGL4.17 was linearised and the confirmed human PEPCK gene promoter sequence isolated from TOPO2.1 using SacI and XhoI restriction enzymes. The PCR fragment was ligated into the cut pGL4.17 vector and positive clones propagated in competent *E.Coli*. The pGL4.17 containing the human PEPCK plasmid was confirmed by in house sequencing (Appendix 2).

The pGL4.17 human PEPCK construct (Figure 4.2) was then transfected into H4IIe rat human hepatoma cells using the calcium phosphate precipitation method (Chapter 2.2.19). The cells were cultured in the presence of neomycin to select for transfected cells only as pGL4.17 includes a neomycin resistance gene. Once colonies of resistant H4IIe cells were established, they were isolated and cultured separately to obtain large numbers of cells. Four such colonies were chosen for characterisation (CSHP 1,2,8 and 12 = Chris Schofield Human PEPCK and denotes who undertook the transfection and selection of colonies along with the gene promoter). Cells from all colonies were frozen at -80 $^{\circ}$C and transferred to liquid nitrogen for long term storage (Chapter 2.2.2.3).

4.2.2.2. Characterisation of CSHP Cells

To assess whether the stably inserted recombinant human PEPCK promoter luciferase reporter was under normal hormonal control, all four colonies of CSHP cells were

plated and allowed to reach 60% confluence. The cells were fasted for 3 hours prior to treatment for 16 hours with serum free media or stimulation with dexamethasone (500 nM) and the second messenger cAMP (100 μM 8-CPT cAMP) in the presence or absence of increasing concentrations of insulin. The cells were lysed and assayed for luciferase activity (Chapter 2.2.20) All colonies showed a stimulation of luciferase activity with dexamethasone and cAMP and this was dominantly repressed by insulin (Figure 4.8).

The fold change by the addition of dexamethasone and cAMP ranged from 4.5 times for colony 8 to 5.6 times for colonies 1 and 12 (Figure 4.9 A). The percentage reduction from maximal stimulation by the addition of 10 nM insulin ranged from 65% for colony 12 to 80% for colony 2 (Figure 4.9 B). Therefore it was decided to use CSHP12, for further characterisation of insulin signalling to the recombinant gene and in the development of an insulin resistant model.

PEPCK gene transcription is regulated within minutes of cellular exposure to hormones, and changes in mRNA are evident between 1 and 3 hours after exposure. Hence, to investigate the optimal exposure time for assessment of luciferase activity regulation, CSHP12 cells were further investigated by varying the length of time they were exposed to hormones. Cells were fasted for 3 hours and then treated for either 3, 8 or 16 hours with dexamethasone and cAMP in the presence or absence of 1nM insulin. At 3 hours stimulation of the cells with dexamethasone and cAMP there was no significant stimulation of luciferase activity with a fold change of 0.9 (Figure 4.10 A and B). As there was no stimulation, there was also no dominant repression by insulin. After 8 hours, there was still minimal stimulation of luciferase activity with dexamethasone and

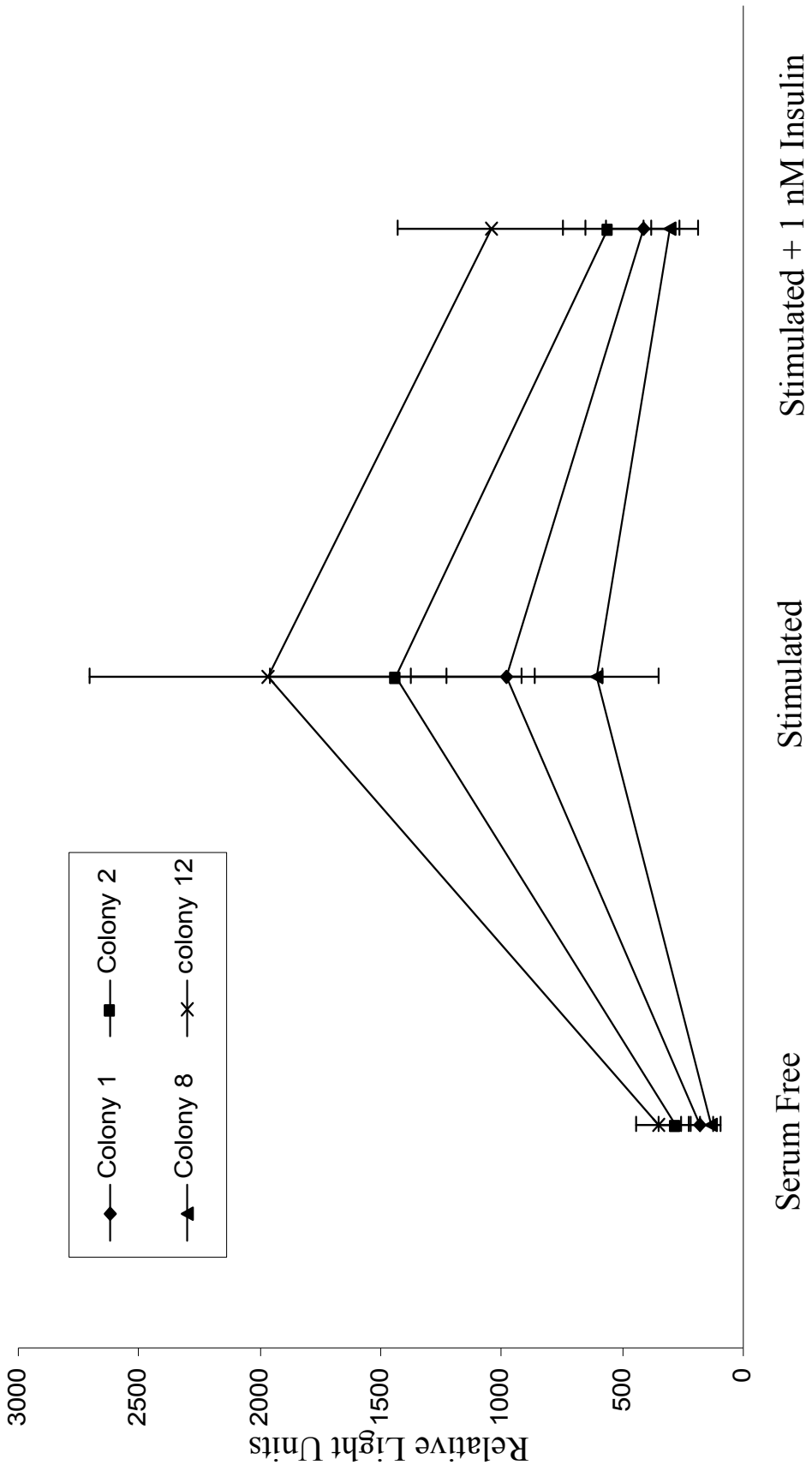


Figure 4.8 Colony testing of H4IIe cells stably transfected with pGL4.17 human PEPCK. Cells were starved for 3 hours then treated for 16 hours with Dexamethasone and cyclic AMP in the presence or absence of insulin before lysis and assay of luciferase activity (n=4)

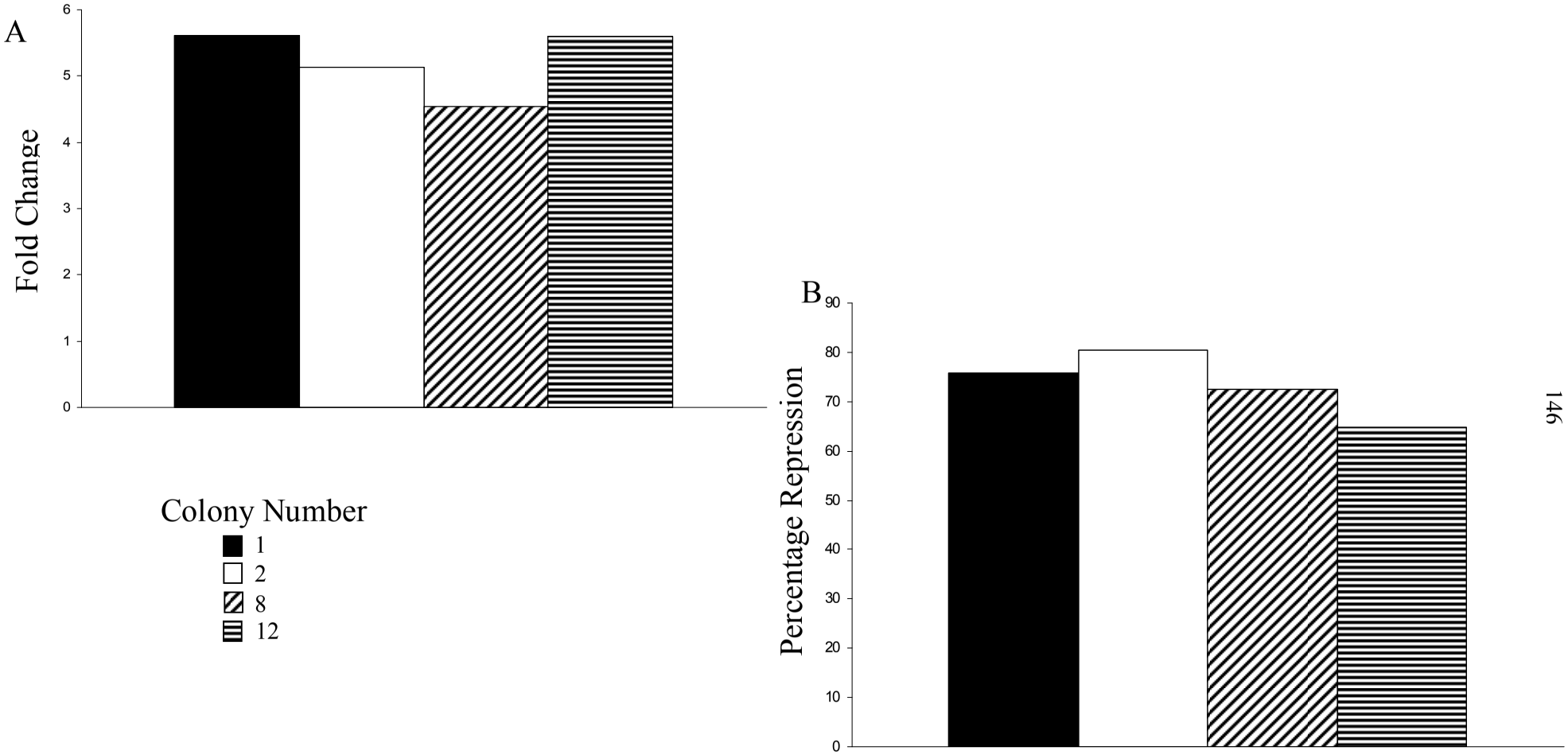
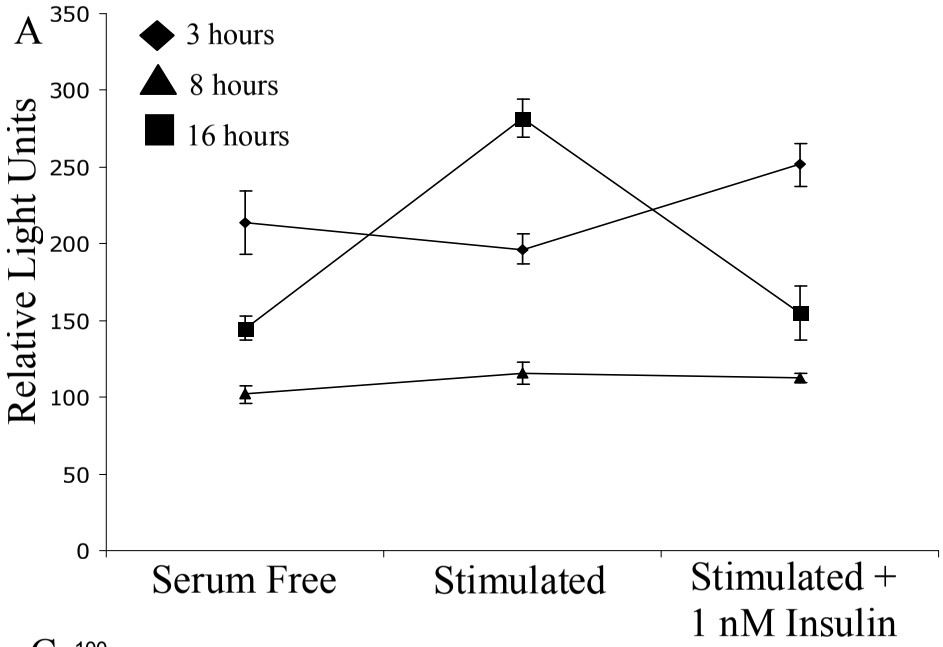


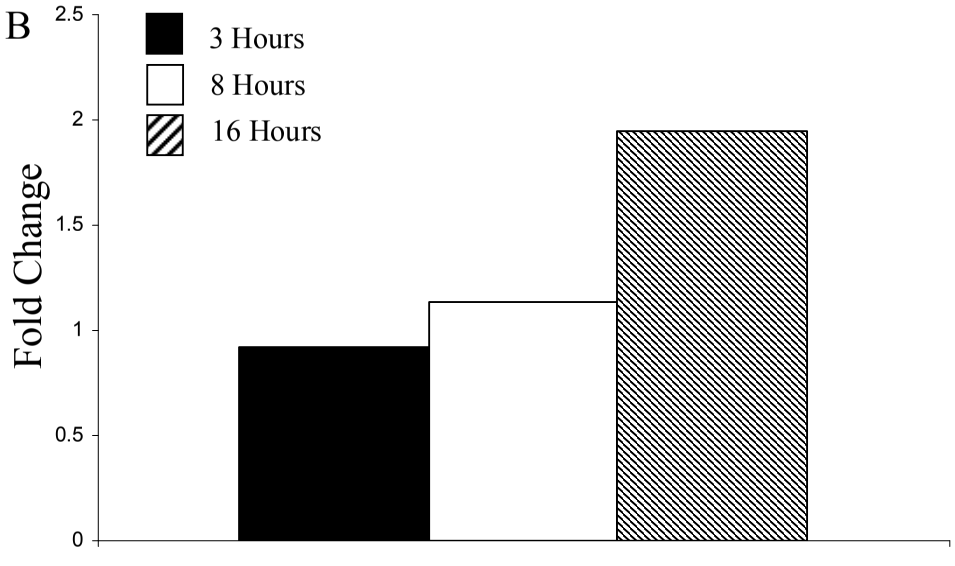
Figure 4.9 Testing of H4IIe cells stably transfected with pGL4.17 Human PEPCK. (A) Fold stimulation of relative light units from basal levels by the addition of Dexamethasone and cyclic AMP. (B) Percentage repression of relative light units from maximal stimulation with dexamethasone and cyclic AMP by the addition of 10 nM Insulin. (n=4)

cAMP, with a fold change of 1.1 (Figure 4.10 A and B). Despite very little stimulation, there was only a 21% reduction in luciferase activity by insulin (Figure 4.10 C). At 16 hours there was a 2-fold change in luciferase activity expression with the addition of dexamethasone and cAMP and a 93% reduction with the addition of 1 nM insulin (Figure 4.10 A, B and C). Therefore, all further experiments involved exposure to hormones for 16 hours unless otherwise stated.

Luciferase activity in LLRP7 cells was shown to be increased by culturing in increasing doses of insulin. Therefore, the effect of insulin alone on luciferase expression was assessed in CSHP12 cells. Cells were fasted for 3 hours prior to incubation with 0.1, 1 or 10 nM insulin for 16 hours before lysis and assay of luciferase activity. In contrast to LLRP7 cells there was a repression of luciferase expression below basal by the addition of 0.1, 1 or 10 nM insulin (Figure 4.11). This could mean that the enhancement in LLRP7 is due to site of insertion in the genome, or there are different regulatory elements in the human PEPCK gene promoter. Further experiments were performed in the presence of 1 nM insulin to maintain constant conditions between each reporter cell.

In order to establish whether the recombinant gene in CSHP12 cells maintained the same signalling connections downstream of the insulin receptor (see Chapter 1.6 and1.11 for details) the cells were fasted for 3 hours prior to 30 minutes pre-incubation with the GSK-3 inhibitor CT99021, the PI 3-K inhibitor LY294002 or the PKB inhibitor Akti1/2 followed by stimulation with dexamethasone (500 nM) and the second messenger cAMP (100μM 8-CPT cAMP) in the presence or absence of 1 nM insulin for 16 hours (Figure 4.12). The inhibition of GSK-3 had no effect on the ability of dexamethasone and cAMP to stimulate, or insulin to inhibit, luciferase activity (Figure 4.12). The addition of LY294002 increased the stimulatory effect of dexamethasone and





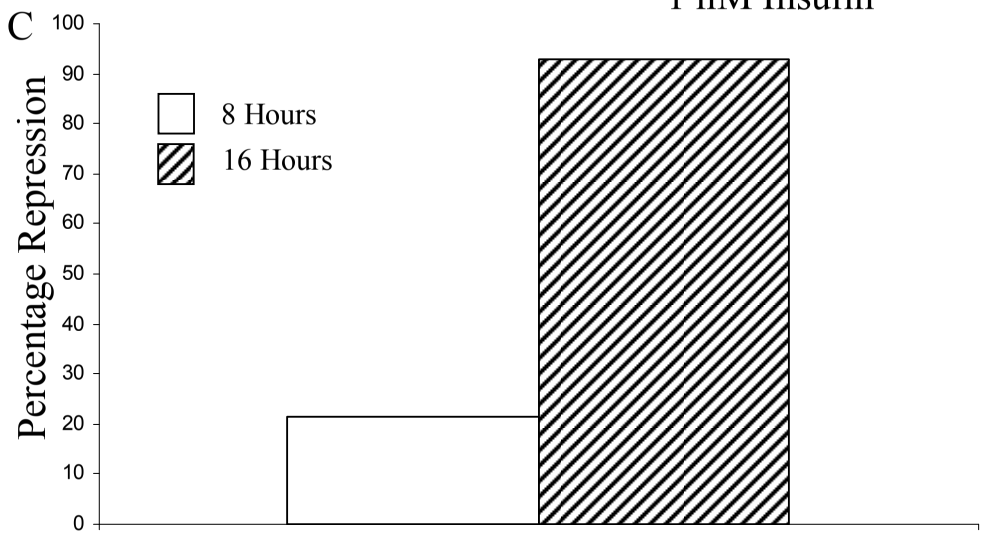


Figure 4.10 Testing of colony 12 H4IIe cells stably transfected with pGL4.17 human PEPCK (A) Cells were starved for 3 hours then treated for differing times with Dexamethasone and cyclic AMP in the presence or absence of insulin before lysis and assay of luciferase activity. Treatment times, Diamonds = 3 hours, Squares = 8 hours, Circles = 16 hours. (n=2)
(B) Fold stimulation of relative light units from basal levels by the addition of Dexamethasone and cyclic AMP at differing time periods
(C) Percentage repression of relative light units from maximal stimulation with dexamethasone and cyclic AMP by the addition of 1 nM Insulin at differing time periods.

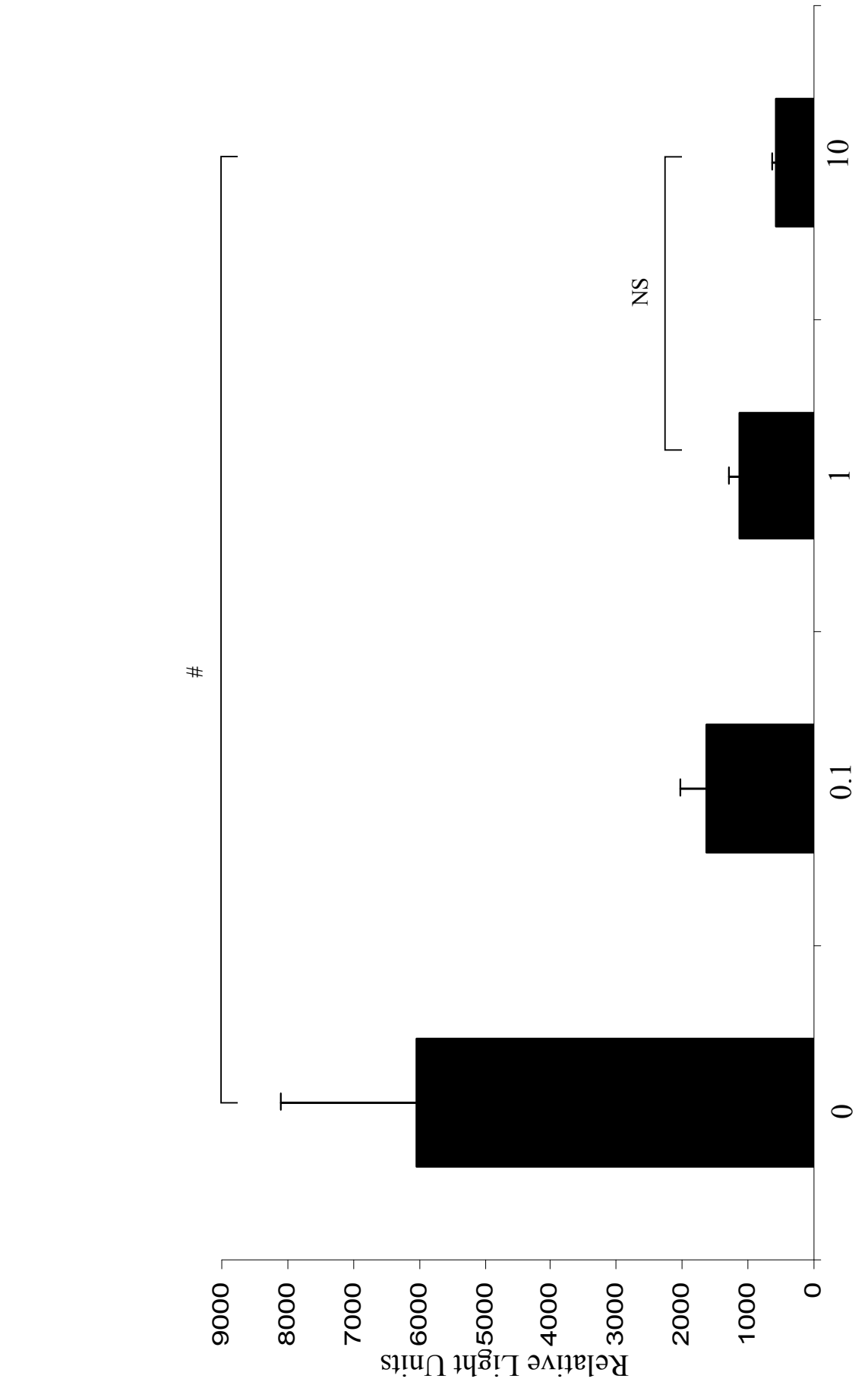


Figure 4.11 Effect of insulin on luciferase activity in CSHP12 cells. Cells were fasted for 3 hours prior to incubation with insulin as indicated before lysis and measurement of luciferase activity. (n=2). P values NS=nonsignificant, #=0.05

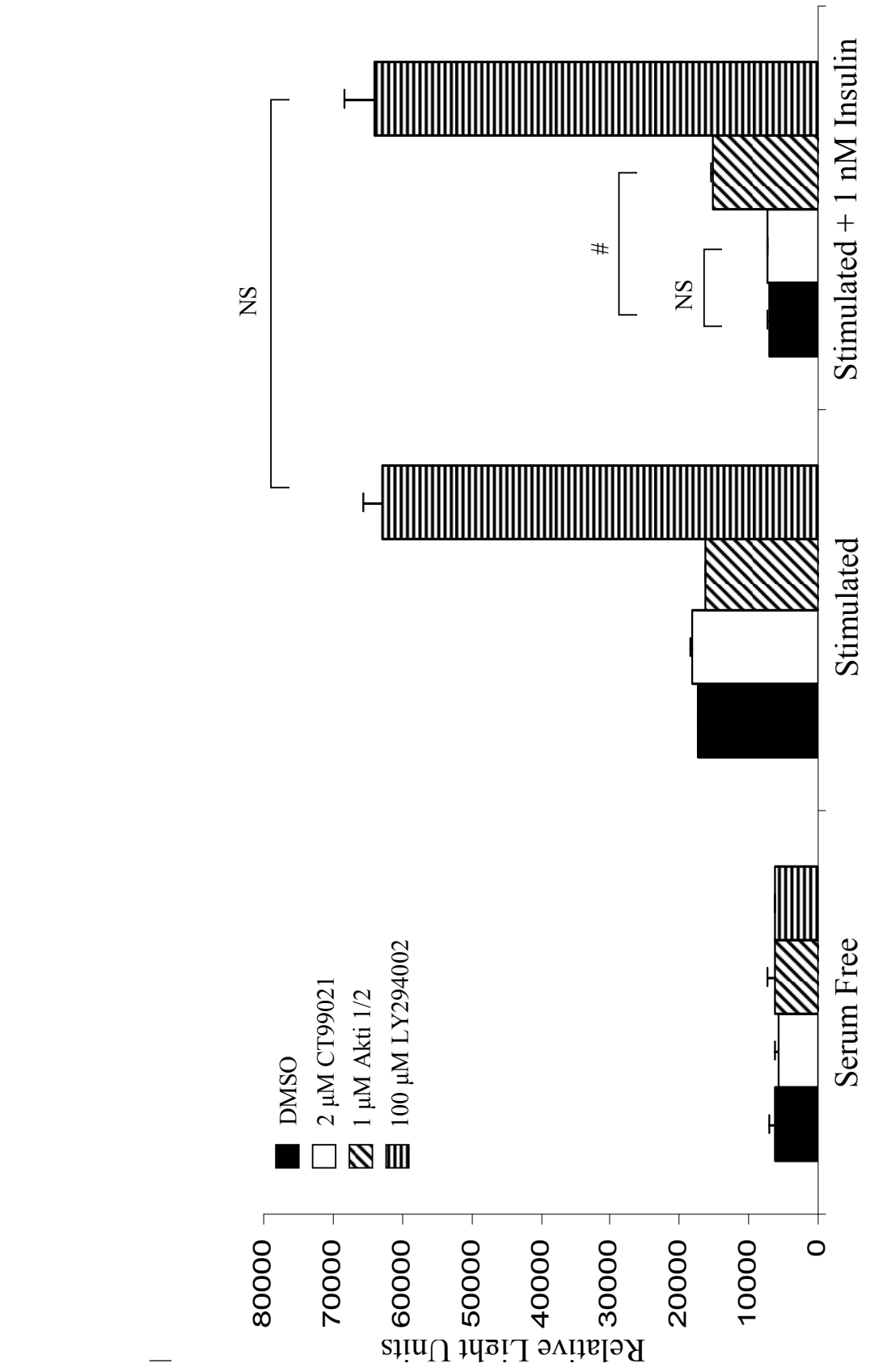


Figure 4.12 Effect of inhibition of GSK-3 by CT99021, PKB by Akti 1/2 and PI 3-K by LY294002 on H4IIe cells stably transfected with pGL4.17 Human PEPCK. Cells were starved for 3 hours and then pre-treated for 30 minutes with inhibitors as indicated followed by treatment for 16 hours in the presence or absence of inhibitors as indicated (n=2). P values NS=non significant, #=0.001

cAMP on luciferase activity and prevented insulin mediated repression (Figure 4.12). Inhibition of PKB had no effect on stimulation, but completely blocked any insulin mediated repression of luciferase activity (Figure 4.12).

In summary, CSHP12 cells are a stably transfected reporter cell line that express luciferase under the control of 3 kbp of the human PEPCK gene promoter. The reporter, like the endogenous gene, is responsive to stimulation by dexamethasone and cAMP, and is dominantly repressed by the addition of insulin. This is most robust with 16 hours of hormone treatments. Insulin signalling connecting the receptor to the recombinant gene promoter appears similar to that reported for the endogenous gene promoter since inhibition of insulin signalling pathway at the level of PKB or PI 3-K prevents the insulin repression of PEPCK. However it differs at other points as GSK-3 inhibition should represses PEPCK transcription in this cell line but does not.

4.2.3. Human G6Pase gene promoter-luciferase reporter cells

4.2.3.1. Production of reporter cells with human G6Pase promoter

Firstly, 2870 bp of the human G6Pase promoter were cloned by PCR from human genomic DNA using primers designed to include 85 bp 3' of the TSS and 2785 bp 5' of the TSS, as shown (Table 4.1, Figure 4.1 and 4.2). The PCR product was purified by agarose gel electrophoresis and ligated into the TOPO 2.1 subcloning vector prior to full sequencing (Chapter 2.2.13). The vector pGL4.17 was linearised and the confirmed human G6Pase sequence isolated from TOPO 2.1 using KpnI and XhoI restriction enzymes. The PCR fragment was ligated into the cut pGL4.17 vector and positive clones propagated in competent *E.Coli*. The pGL4.17 containing the human G6Pase plasmid was then confirmed by in house sequencing (Appendix 2).

The pGL4.17 human G6Pase construct (Figure 4.2) was then transfected into H4IIe rat hepatoma cells using the calcium phosphate precipitation method (Chapter 2.2.19). The cells were cultured in the presence of neomycin to select for transfected cells only as pGL4.17 contains a neomycin resistance gene. Once colonies of resistance H4IIe cells were established, they were isolated and cultured. Eight such colonies were chosen for characterisation (LLHG-4.-5,-8,-11,-16,-38,-56,-77 = Lisa Logie Human G6Pase and denotes who undertook the transfection and selection of colonies along with the gene promoter). Cells from all colonies were frozen at -80 °C and transferred to liquid nitrogen for long term storage (Chapter 2.2.2.3).

4.2.3.2. Characterisation of LLHG Cells

To assess whether the stably inserted recombinant human G6Pase promoter luciferase reporter was under normal hormonal control, all 8 colonies of LLHG cells were plated and allowed to reach 60% confluence. The cells were fasted for 3 hours prior to treatment for 16 hours with serum free media or with dexamethasone (500 nM) and the second messenger cAMP (100 μM 8-CPT cAMP) in the presence or absence of increasing concentrations of insulin. The cells were lysed and assayed for luciferase activity (Chapter 2.2.20). All 8 colonies were found to have increased luciferase expression when incubated with dexamethasone and cAMP and in all cases induction was dominantly repressed by insulin (Figure 4.13).

However, the luciferase levels were variable between colonies (Figure 4.13), suggesting that basal expression and regulation were influenced by the position and/or number of gene insertion. The fold change by the addition of dexamethasone and cAMP ranged from 1.8 times for colony 8 to 2.88 times for colony 4 (Figure 4.14 A). The percentage reduction from maximal stimulation by the addition of 10 nM insulin ranged from 54.4% for colony 8 to 148.9% for colony 11 (Figure 4.14 B). It was decided to use LLHG4, for further characterisation of insulin signalling to the recombinant gene.

Differing effects of insulin in the absence of dex/cAMP were obtained on luciferase expression in LLRP7 and CSHP12 cells. Therefore, the effect of insulin alone on luciferase expression was assessed in LLHG4 cells. Cells were fasted for 3 hours prior to incubation with 0.1, 1 or 10 nM insulin for 16 hours before lysis and assay of luciferase activity. There was no increase in luciferase activity with 0.1 or 1 nM insulin (Figure 4.15). However, 10 nM insulin increased luciferase activity by 1.7 fold (Figure

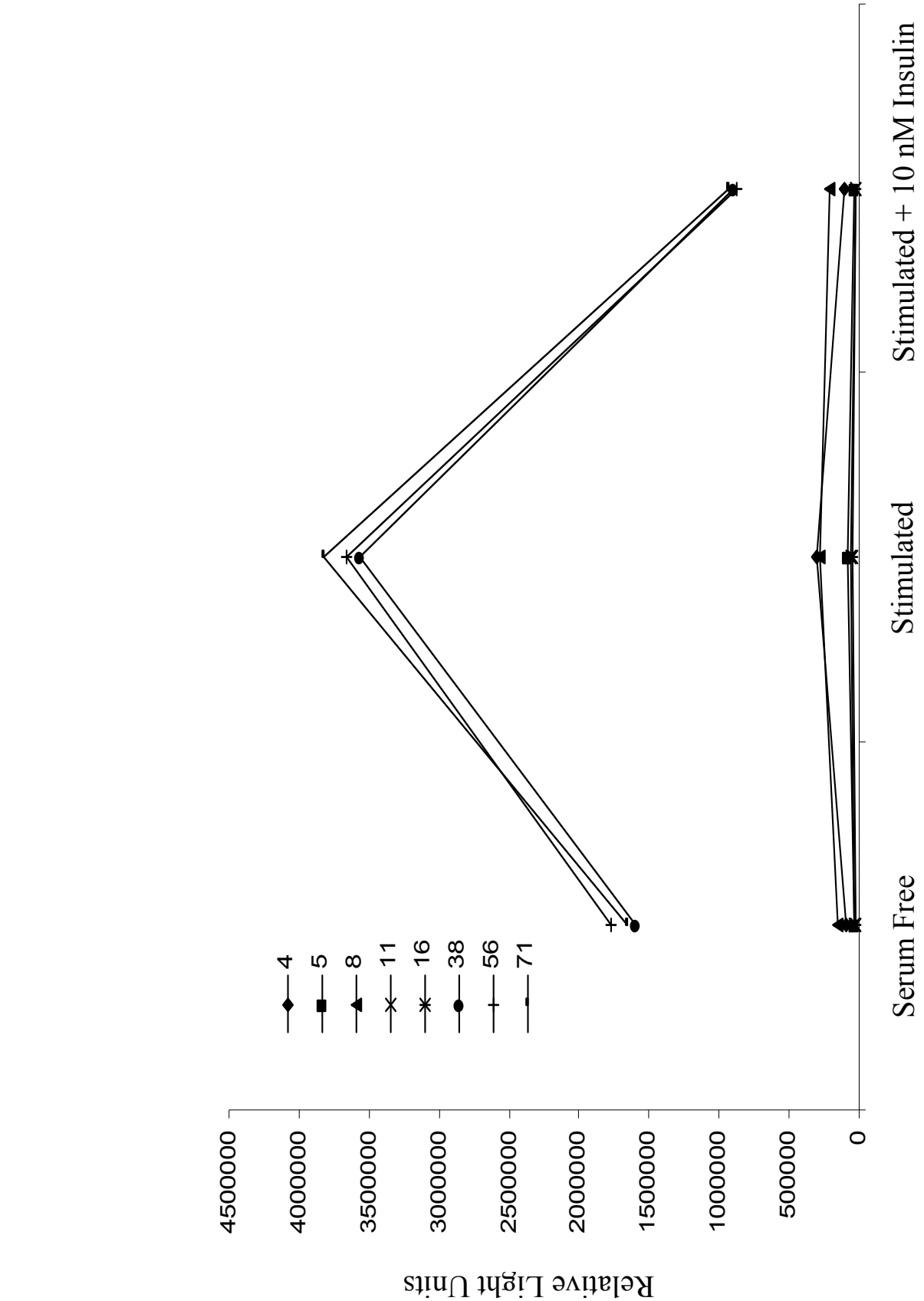
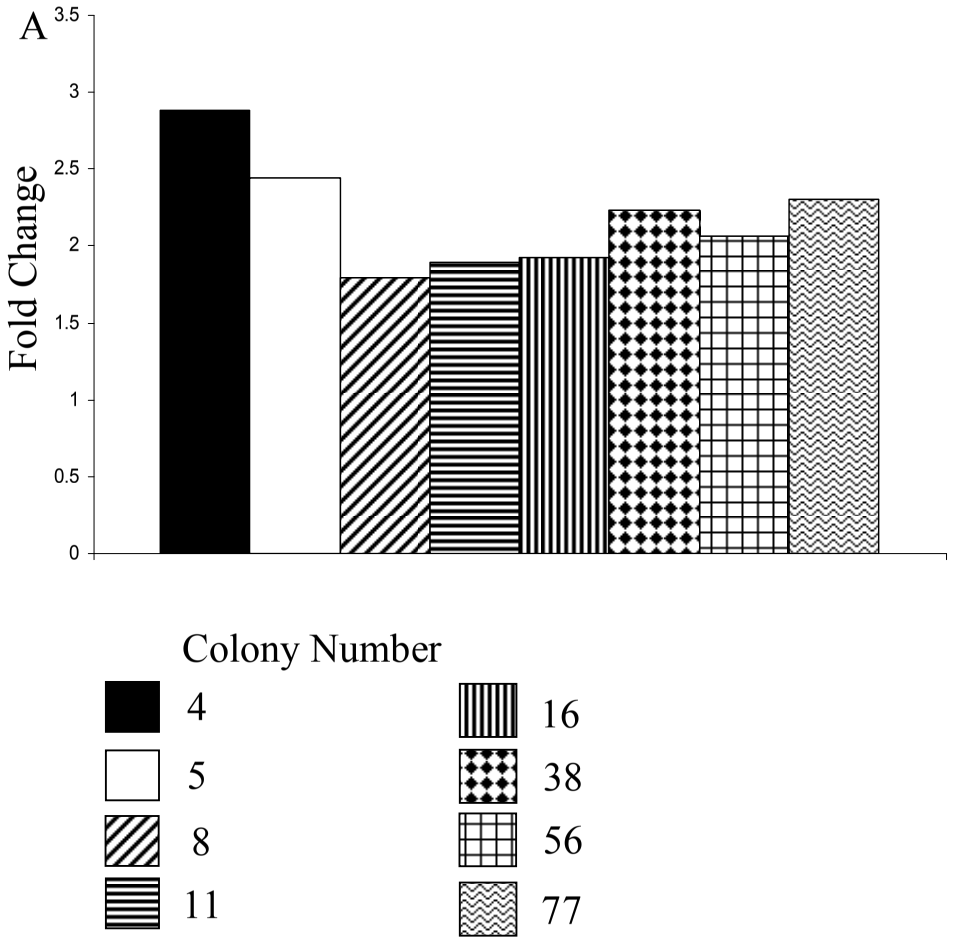


Figure 4.13 Colony testing of H4IIe cells stably transfected with pGL4.17 human G6Pase. Cells were starved for 3 hours then treated for 16 hours with Dexamethasone and cyclic AMP in the presence or absence of insulin before lysis and assay of luciferase activity. All tests performed in duplicate and shown as mean relative light units (n=2-4).



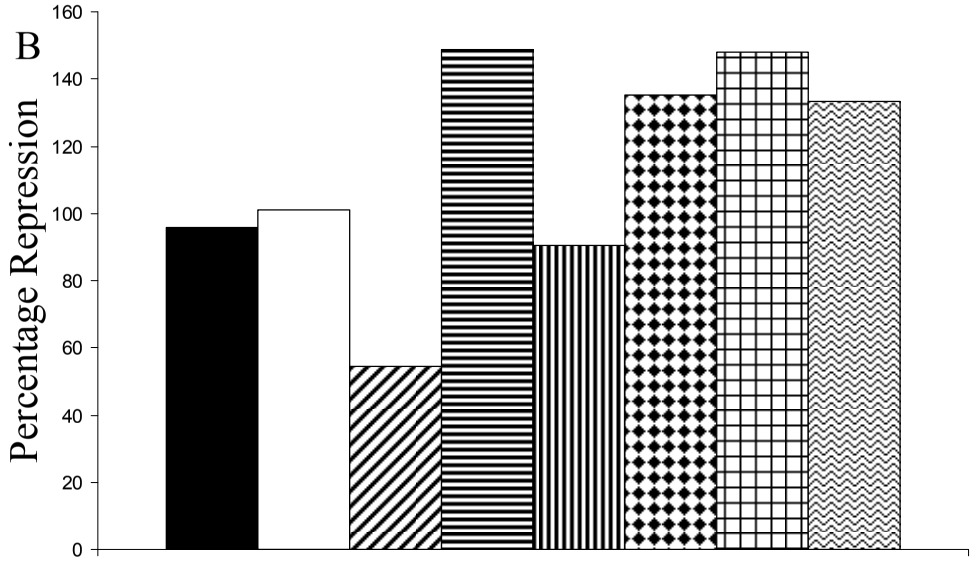


Figure 4.14 Testing of H4IIe cells stably transfected with pGL4.17 Human G6Pase. (A) Fold stimulation of relative light units from basal levels by the addition of Dexamethasone and cyclic AMP. (B) Percentage repression of relative light units from maximal stimulation with dexamethasone and cyclic AMP by the addition of 10 nM Insulin (n=2-4).

4.15). Further experiments were performed in the presence of 1 nM insulin to prevent this confounding effect.

Regulation of the endogenous G6Pase gene promoter by insulin involves the PI 3-K, PKB and GSK-3 signalling pathway (see Chapter 1.6 and 1.11 for details). In order to establish whether the recombinant gene in LLHG4 cells maintained the same signalling connections downstream of the insulin receptor, cells were fasted for 3 hours prior to 30 minutes pre-incubation with the GSK-3 inhibitor CT99021, the PI 3-K inhibitor LY294002 or the PKB inhibitor Akti 1/2, followed by 16 hours stimulation with dexamethasone (500 nM) and the second messenger cAMP (100 μM 8-CPT cAMP) in the presence or absence of 1 nM insulin (Figure 4.16). Pharmacological inhibition of PI 3-K by LY294002 increased the stimulation of luciferase activity by dexamethasone and cAMP and prevented its subsequent repression by insulin (Figure 4.16) consistent with its effects on G6Pase gene transcription. The pharmacological inhibition of GSK-3 had no effect on either stimulation or repression of luciferase activity (Figure 4.16). The PKB inhibitor reduced the repressive effects of insulin on luciferase activity consistent with the effects seen on the endogenous G6Pase gene promoter (Figure 4.16).

In summary, LLHG4 cells are a stably transfected reporter cell line that expresses luciferase under the control of 2.8kbp of the human G6Pase gene promoter. The reporter, like the endogenous gene is responsive to stimulation with dexamethasone and cAMP, and luciferase activity is dominantly repressed by the addition of insulin. Insulin signalling connecting the receptor to the recombinant gene promoter, appears similar to that reported for the endogenous gene promoter since inhibition of PI 3-K and

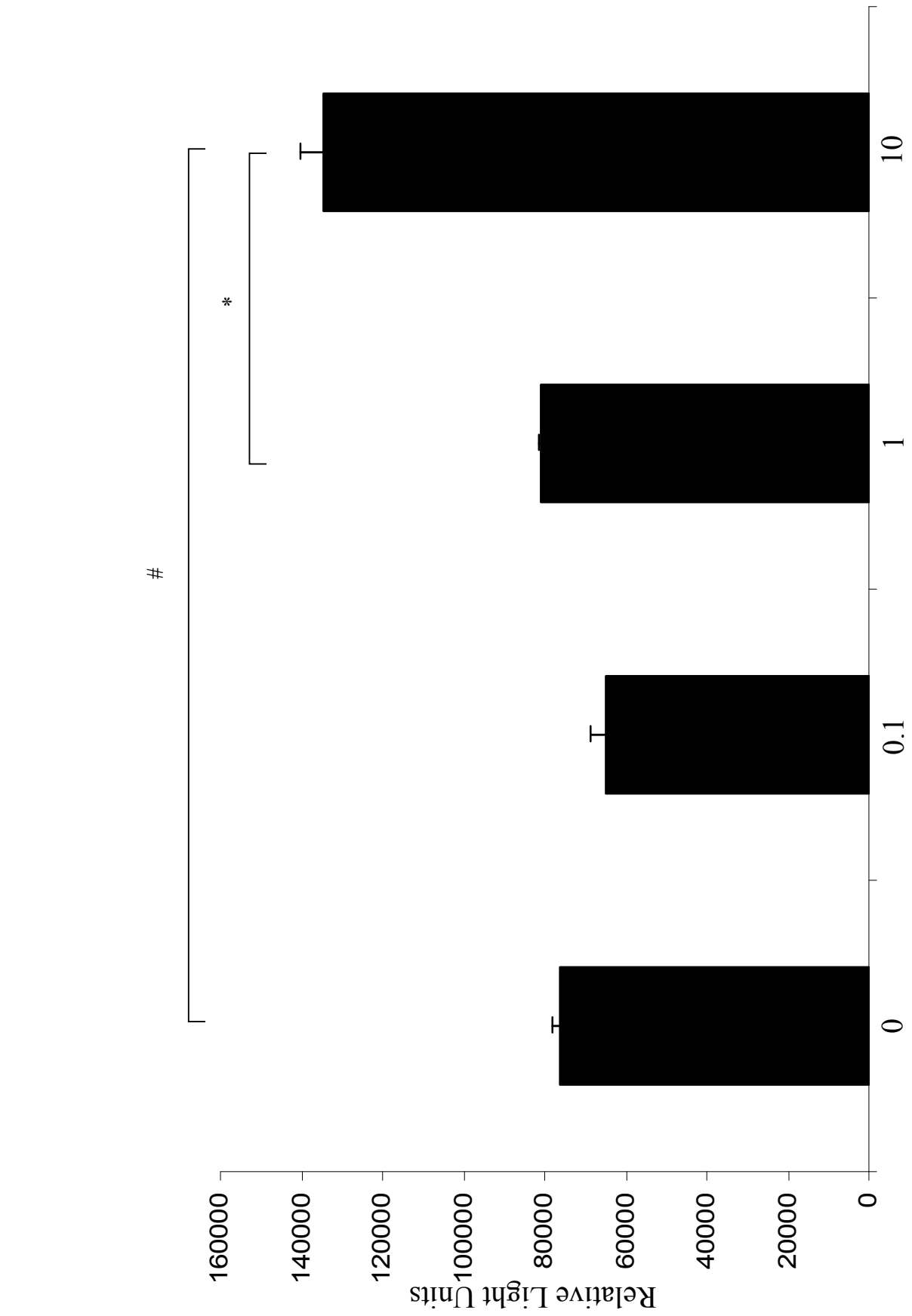


Figure 4.15 Effect of insulin on luciferase activity in LLHG4 cells. Cells were fasted for 3 hours prior to incubation with insulin as indicated before lysis and measurement of luciferase activity (n=2). P values, #=0.005, *=0.009

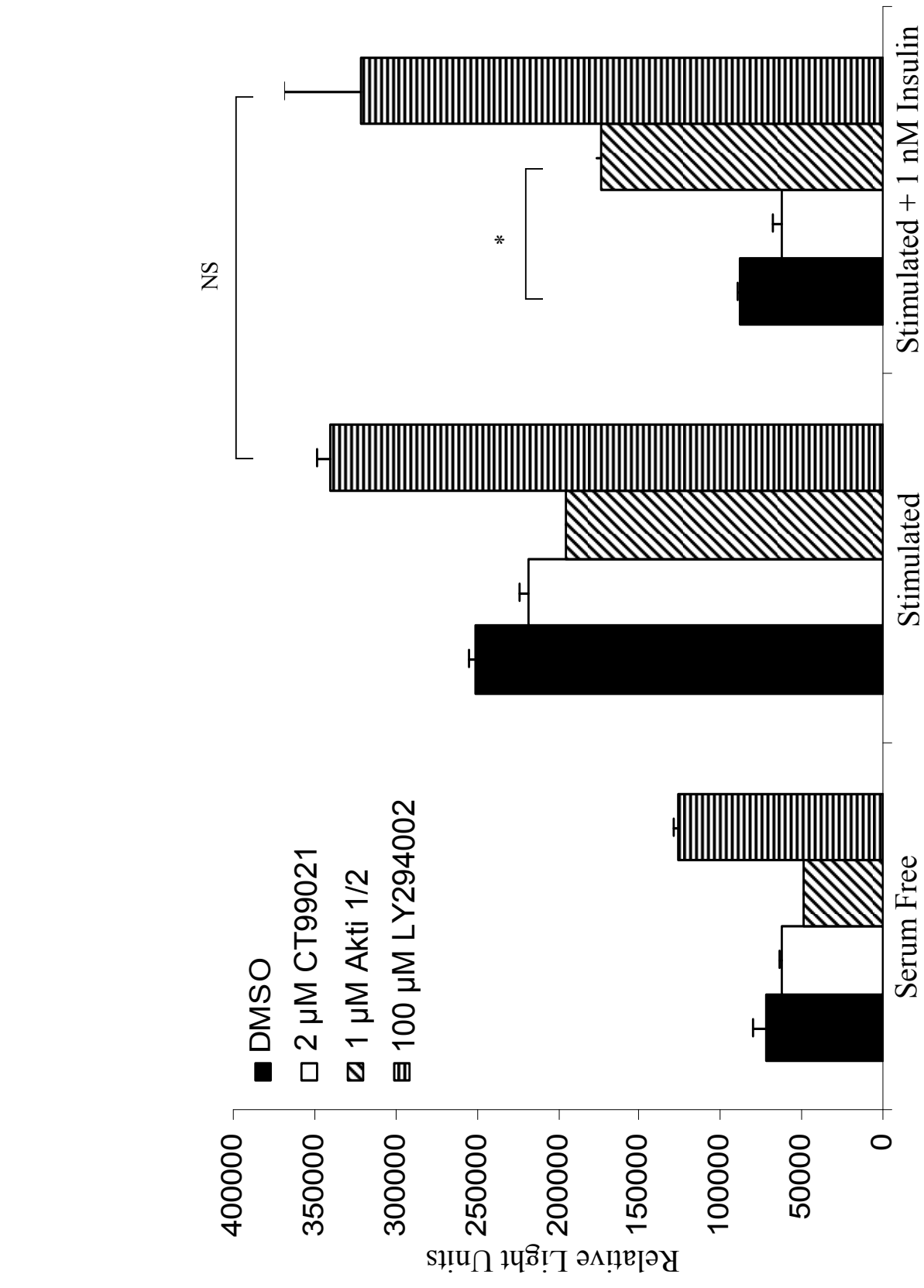


Figure 4.16 Effect of inhibition of GSK-3 by CT99021, PI 3-K by LY294002 and PKB by Akti 1/2. LLHG4 cells were fasted for 3 hours before pre-treatment with indicated inhibitors for 30 minutes. Cells were then treated for 16 hours as indicated (n=2).P values, NS=non significant, *=<0.001

PKB prevents the repression of reporter production by insulin. However, GSK-3 inhibition represses endogenous G6Pase transcription but in this reporter cell line it does not.

4.2.4. Human IGFBP-1 promoter-luciferase reporter cells

4.2.4.1. Production of CSHI cells

Firstly, 3186 bp of the human IGFBP-1 promoter were cloned by PCR from human genomic DNA using primers designed to include 229 bp 3' of the TSS and 2957 bp 5' of the TSS, as shown (Table 4.1, Figure 4.1 and 4.2). The resulting PCR product was purified by agarose gel electrophoresis prior and ligated into the TOPO 2.1 subcloning vector prior to full sequencing (Ch2.2.13). The vector pGL4.17 was linearised and the confirmed human IGFBP-1 gene promoter sequence isolated from TOPO2.1 using SacI and XhoI restriction enzymes. The PCR fragment was ligated into the cut pGL4.17 vector and positive clones propagated in competent *E.Coli*. The pGL4.17 containing the human IGFBP-1 gene promoter was confirmed by in house sequencing (Appendix 2).

pGL4.17 human IGFBP-1 construct (Figure 4.2) was then transfected into H4IIe human hepatoma cells using the calcium phosphate precipitation method (Chapter 2.2.19). The cells were cultured in the presence of neomycin to select for transfected cells only as pGL4.17 includes a neomycin resistance gene. Once colonies of resistant H4IIe cells were established, they were isolated and cultured separately. Six such colonies were chosen for characterisation (CSHI 1-6 = Chris Schofield Human IGFBP-1 and denotes who undertook the transfection and selection of colonies along with the gene promoter). Cells from all colonies were frozen at -80 °C and transferred to liquid nitrogen for long term storage (Chapter 2.2.2.3).

4.2.4.2. Characterisation of CSHI Cells

In an attempt to assess whether the stably inserted recombinant human IGFBP-1 promoter luciferase reporter was under normal hormonal control, all six colonies of CSHI cells were plated and allowed to reach 60% confluence. The cells were fasted for 3 hours prior to 16 hours treatment with serum free media or with dexamethasone (500 nM) and the second messenger cAMP (100 μM 8-CPT cAMP) in the presence or absence of increasing concentrations of insulin. The cells were lysed and assayed for luciferase activity (Chapter 2.2.20). All six colonies were stimulated by dexamethasone and cAMP and in all cases the induction of luciferase activity dominantly repressed by insulin (Figure 4.17).

The levels of luciferase were variable between colonies (Figure 4.17), suggesting that the basal expression and regulation of luciferase were influenced by the number and position of gene insertion within the genome. The fold change in luciferase activity by the addition of dexamethasone and cAMP ranged from 5.9 times for colony 1 to 27.0 times for colony 4 (Figure 4.18). The percentage reduction from maximal stimulation by the addition of 10 nM insulin ranged from 33.15% for colony 5 to 82% for colony 1 (Figure 4.18). Colony 4 showed a fold change of 27.0 from basal levels and a repression of 61.9% by the addition of 10 nM insulin (Figure 4.18). Therefore CSHI-4 cells were chosen for further characterisation.

As with the other gene promoter constructs, it was essential to assess the most robust conditions under which to perform the assay. Thus, to investigate the optimal exposure

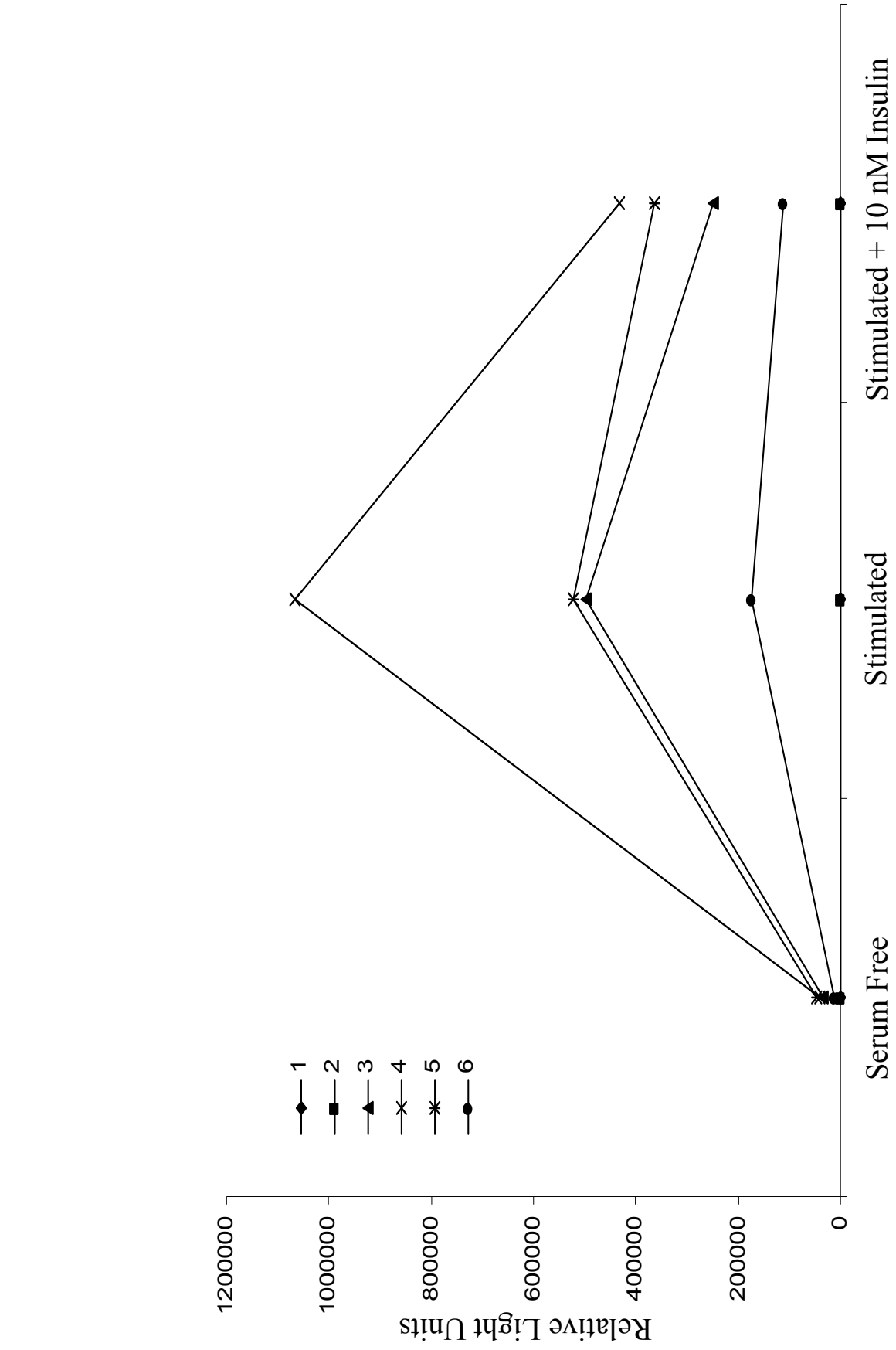


Figure 4.17 Colony testing of H4IIe cells stably transfected with pGL4.17 human IGFBP-1. Cells were starved for 3 hours then treated for 16 hours with Dexamethasone and cyclic AMP in the presence or absence of insulin before lysis and assay of luciferase activity. (n=2-6 shown as mean)

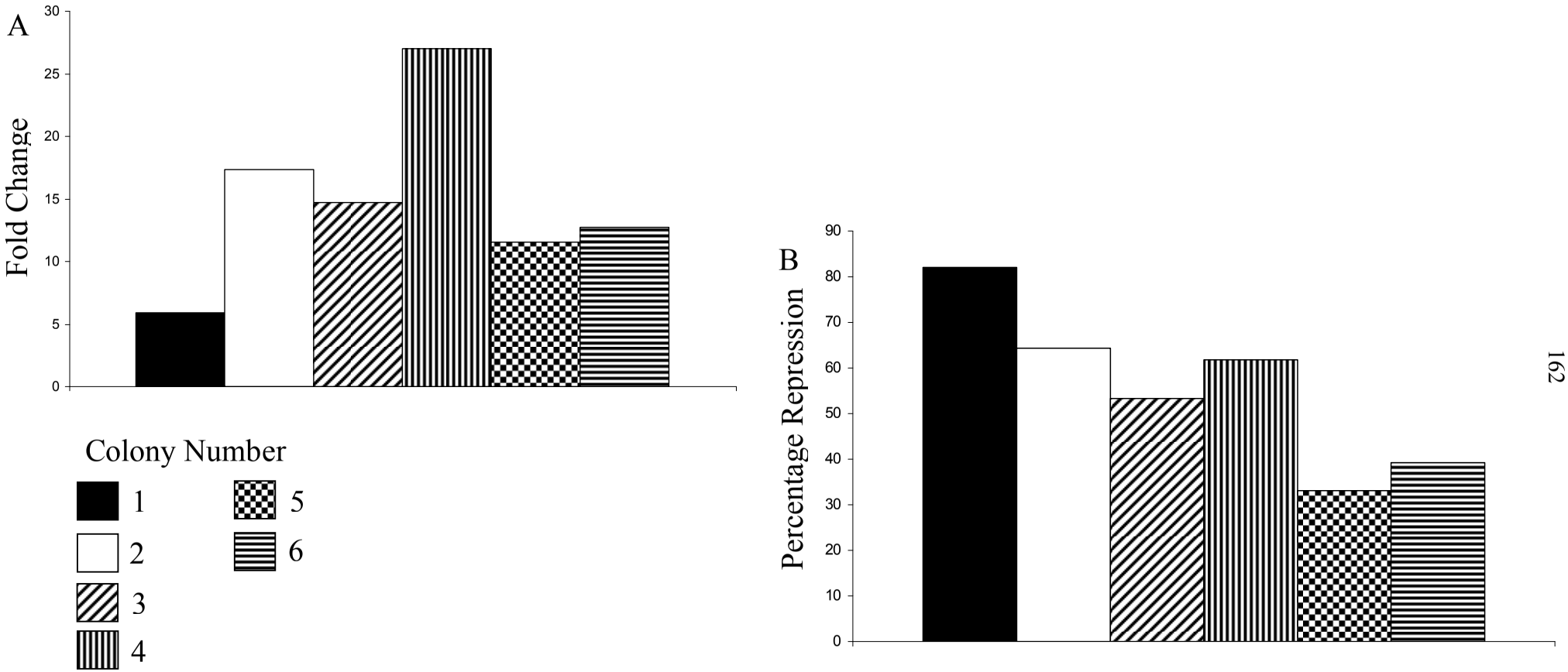


Figure 4.18 Testing of H4IIe cells stably transfected with IGFBP-1. (A) Fold stimulation of relative light units from basal levels by the addition of Dexamethasone and cyclic AMP. (B) Percentage repression of relative light units from maximal stimulation with dexamethasone and cyclic AMP by the addition of 10 nM Insulin (n=2-4).

time for assessment of luciferase activity regulation, CSHI-4 cells were further investigated by varying the length of exposure to hormones. Cells were fasted for 3 hours and then treated for either 3, 8 or 16 hours with dexamethasone and cAMP in the presence or absence of 1nM insulin. At 3 hours there was no significant stimulation of IGFBP-1 with dexamethasone and cAMP (Figure 4.19). However, at 8 hours there was stimulation of luciferase activity by dexamethasone and cAMP 6.7 times above basal (Figure 4.19) and the percentage repression by 1 nM insulin was 17.8% (Figure 4.19). At the sixteen hour time point the fold change in luciferase activity with the addition of dexamethasone and cAMP was 34.4 times (Figure 4.19) and a 49.6% reduction with the addition of 1 nM insulin (Figures 4.19). Therefore, all further experiments involved hormone exposure for 16 hours unless stated.

As with the other cell lines I wished to assess the effects of insulin on luciferase activity in the absence of dex/cAMP. CSHI-4 cells were fasted for 3 hours prior to incubation for sixteen hours in either serum free media or serum free media with the addition of 0.1, 1 or 10 nM insulin. The cells were lysed and luciferase activity measured (Chapter 2.2.20). There was no effect of 0.1 nM insulin on luciferase activity. There was a 2.8 fold increase in luciferase activity with 1 nM insulin (Figure 4.20) and a 7.5 fold induction with 10 nM insulin (Figure 4.20). Therefore, it was decided to use 1 nM insulin for further assessment to minimise the enhancement, and maximise the repression by insulin.

Regulation of the endogenous IGFBP-1 gene promoter by insulin involves the PI 3-K, PKB, mTOR and GSK-3 signalling pathways (see Chapter 1.6 and 1.11 for details). In order to establish whether the recombinant gene in CSHI4 cells maintained the same signalling connections downstream of the insulin receptor the cells were fasted

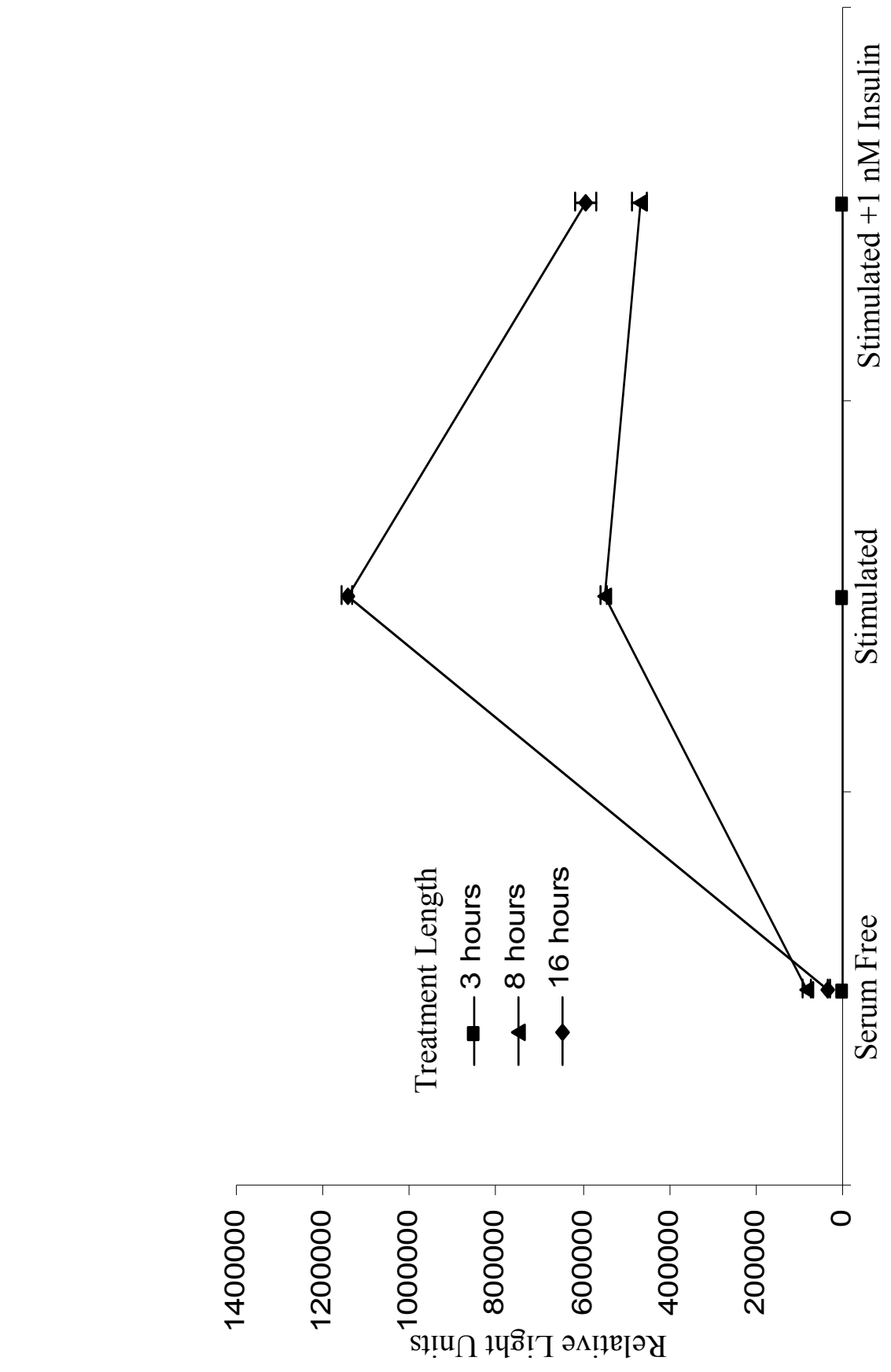


Figure 4.19 Colony testing of H4IIe cells stably transfected with pGL4.17 human IGFBP-1 (CSHI4). Cells were starved for 3 hours then treated for differing times with Dexamethasone and cyclic AMP in the presence or absence of insulin before lysis and assay of luciferase activity (n=2)

for 3 hours prior to 30 minutes pre-incubation with the GSK-3 inhibitor CT99021, the PI 3-K inhibitor LY294002 or the PKB inhibitor Akti 1/2 followed by stimulation with dexamethasone (500 nM) and the second messenger cAMP (100 μM 8-CPT cAMP) in the presence or absence of 10 nM insulin for 16 hours (Figure 4.21). The inhibition of GSK-3 had no effect on the ability of dexamethasone and cAMP to stimulate IGFBP-1 transcription (Figure 4.21). Pharmacological inhibition of PKB did not enhance the stimulatory effect of dexamethasone and cAMP, but did reduce the repressive ability of insulin on luciferase activity (Figure 4.21) consistent with its effects on IGFBP-1 transcription. The pharmacological inhibition of PI 3-K increased the stimulation of luciferase production by glucocorticoids and cAMP and completely blocked the effects of insulin (Figure 4.21). Rapamycin had no effect on the stimulation of luciferase production by glucocorticoids and cAMP and there was no effect on the repressive ability of insulin on luciferase activity (Figure 4.22).

In summary, CSHI4 cells are a stably transfected reporter cell line that expresses luciferase under the control of 3.2 kbp of the human IGFBP-1gene promoter. The reporter, like the endogenous gene is responsive to stimulation by dexamethasone and cAMP, and luciferase activity is dominantly repressed by the addition of insulin. This is most robust with 16 hours of hormone treatment. Higher doses of insulin stimulate luciferase activity. Insulin signalling connecting the receptor to the recombinant gene promoter has some similarities to that reported for the endogenous gene promoter as inhibition of the main insulin signalling pathway at the level of PKB or PI 3-K prevents the insulin repression of IGFBP-1. However GSK-3 inhibition represses endogenous IGFBP-1 transcription and rapamycin can reduce insulin regulation, however this was not observed in this cell line.

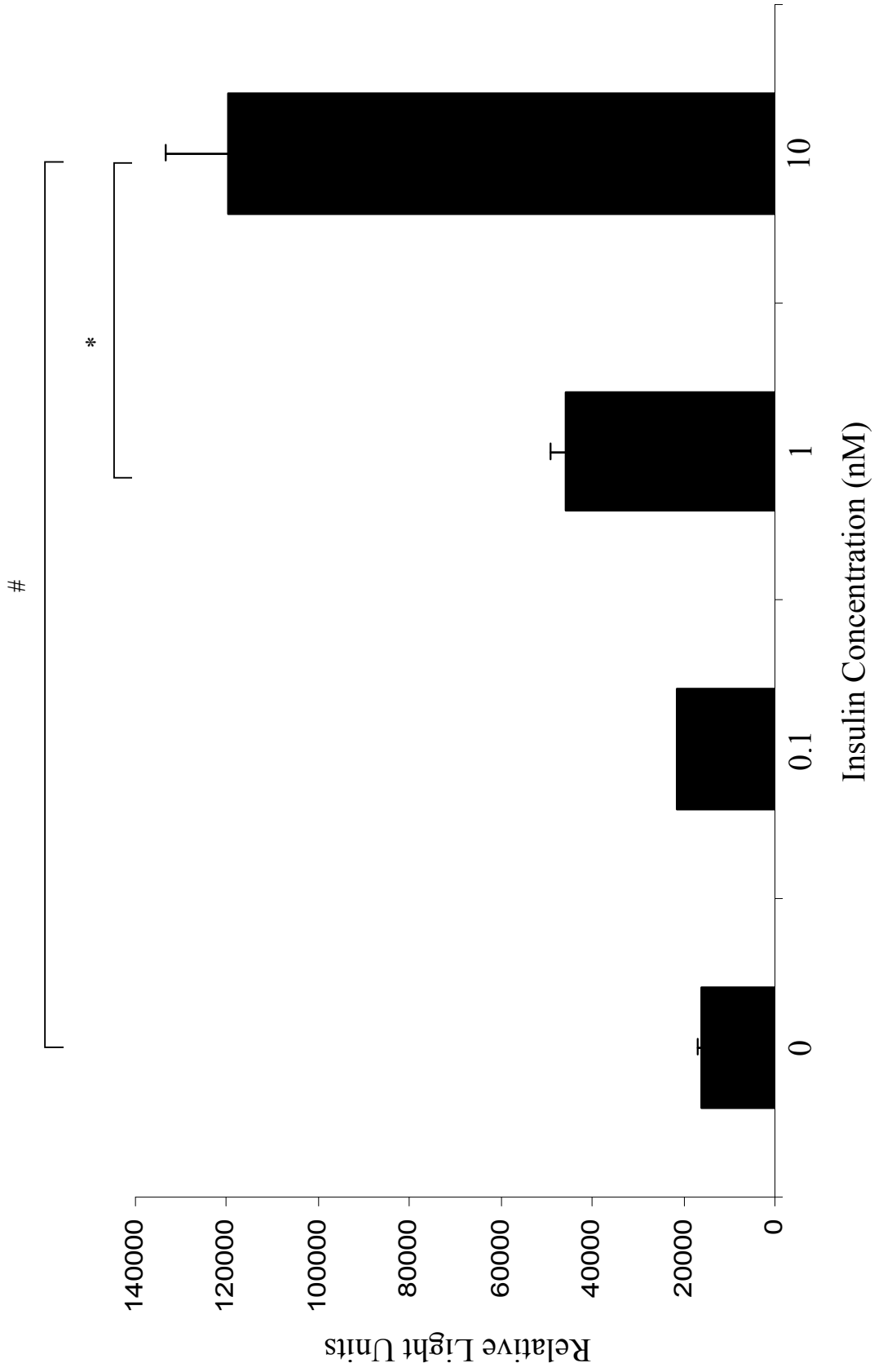


Figure 4.20 Effect of insulin on luciferase activity in CSHI4 cells. Cells were fasted for 3 hours prior to incubation with insulin as indicated before lysis and measurement of luciferase activity (n=2). P values, #=0.008, *=0.03

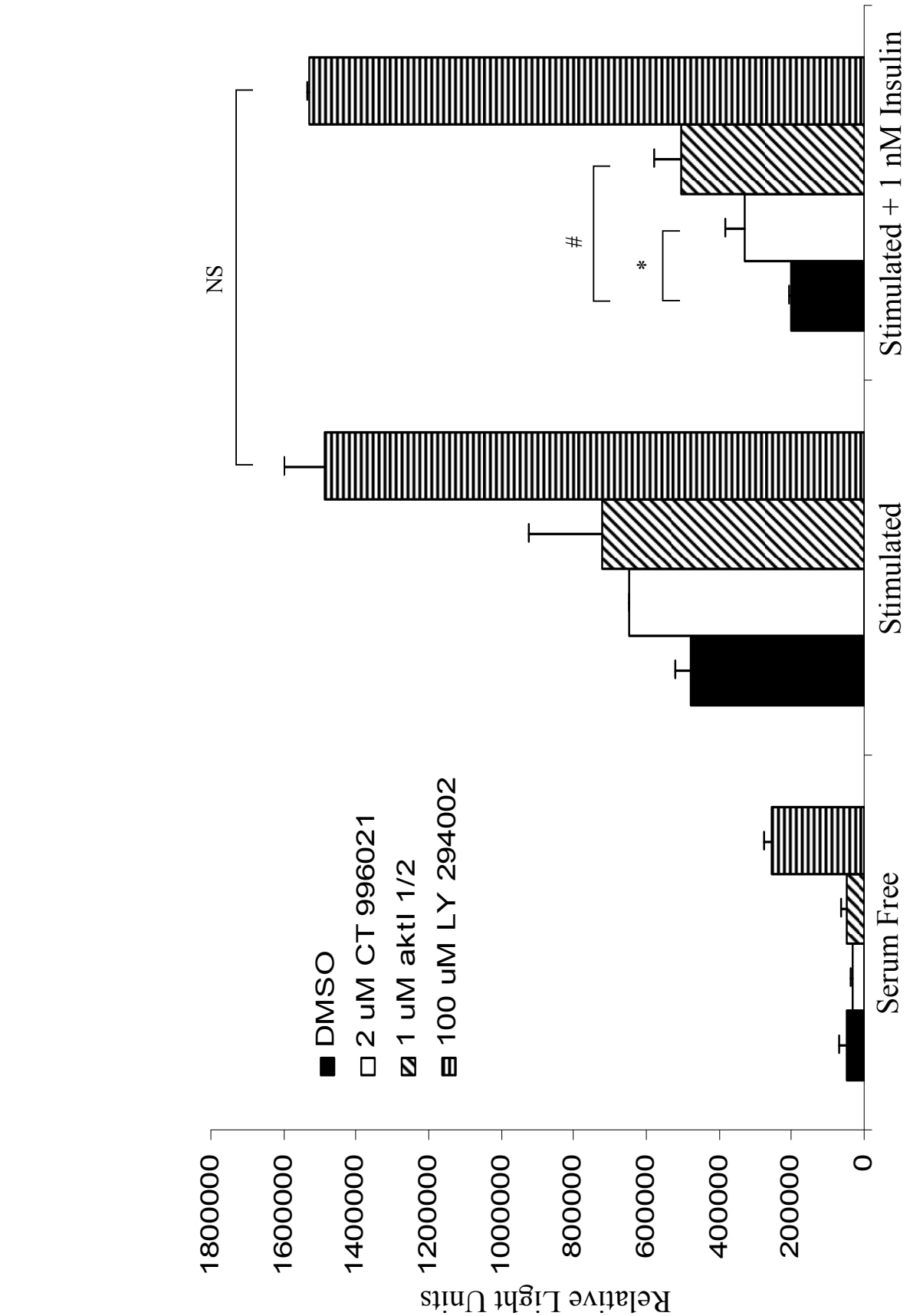


Figure 4.21 Effect of inhibition of GSK-3 by CT99021,PI 3-K by LY294002 and PKB by Akti 1/2 on CSHI-4 cells. Cells were fasted for 3 hours before pre-treatment with indicated inhibitors for 30 minutes. Cells were then treated for 16 hours as indicated. P values, NS=nonsignificant, *=0.06, #=0.05. n=2

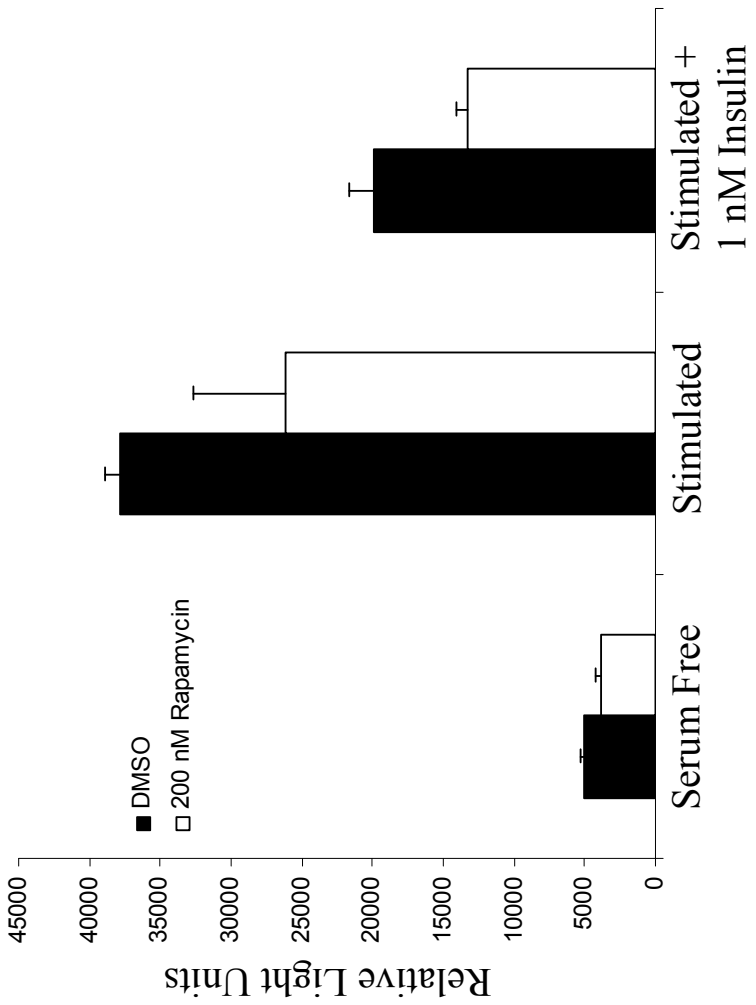


Figure 4.22 Effect of inhibition of mTOR by rapamycin on CSHI-4 cells. Cells were fasted for 3 hours before pre-treatment with indicated inhibitors for 30 minutes. Cells were then treated for 16 hours as indicated (n=2)

4.2.5. Completeness of inhibition by small molecule inhibitors in H4IIe cells

To confirm that H4IIe cells respond to all of the inhibitors used in the reporter cell studies, cells were incubated for 30 minutes in the presence or absence of CT99021, LY294002 or Akti 1/2 for 30 minutes prior to exposure to 10 nM insulin for 1 hour. As expected both the PKB and PI 3-K inhibitor reduced the insulin stimulated Ser473 phosphorylation of PKB (Figure 4.23). Furthermore, inhibition of PI 3-K prevented the phosphorylation of S6 ribosomal protein at Ser240/244, but inhibition of PKB did not (Figure 4.23). The effects of rapamycin on insulin signalling in H4IIe cells are shown in Chapter 5 (Figure 5.23).

4.3. The development of reporter cells as a cell model of insulin resistance

In Chapter 3 I demonstrated that growth of H4IIe cells in serum from humans with diabesity reduced the insulin sensitivity of the cells as measured using insulin repression of PEPCK mRNA. Therefore I attempted to generate similar insulin resistance in the luciferase reporter cells. LLRP7 cells were cultured for 3 weeks in DMEM containing 5% serum from either a control patient (volunteer 2) or a diabetic patient (volunteer 36) (Figure 4.24). The serum from volunteer 36 induced insulin resistance in H4IIe cells (Figures 3.5 and 3.6). After 3 weeks, the cells were fasted for 3 hours prior to stimulation with dexamethasone (500 nM) and the second messenger cAMP (100 μM 8-CPTcAMP) in the presence or absence of increasing concentrations of insulin. After 16 hours the cells were lysed and assayed for luciferase activity. Protein concentration was measured by Bradford assay and luciferase activity was corrected for protein concentration. Unfortunately, for an unknown reason the cells did not respond to dexamethasone and cAMP, making it impossible to examine the insulin sensitivity using the reporter system (Figure 4.24). Due to lack of time and a

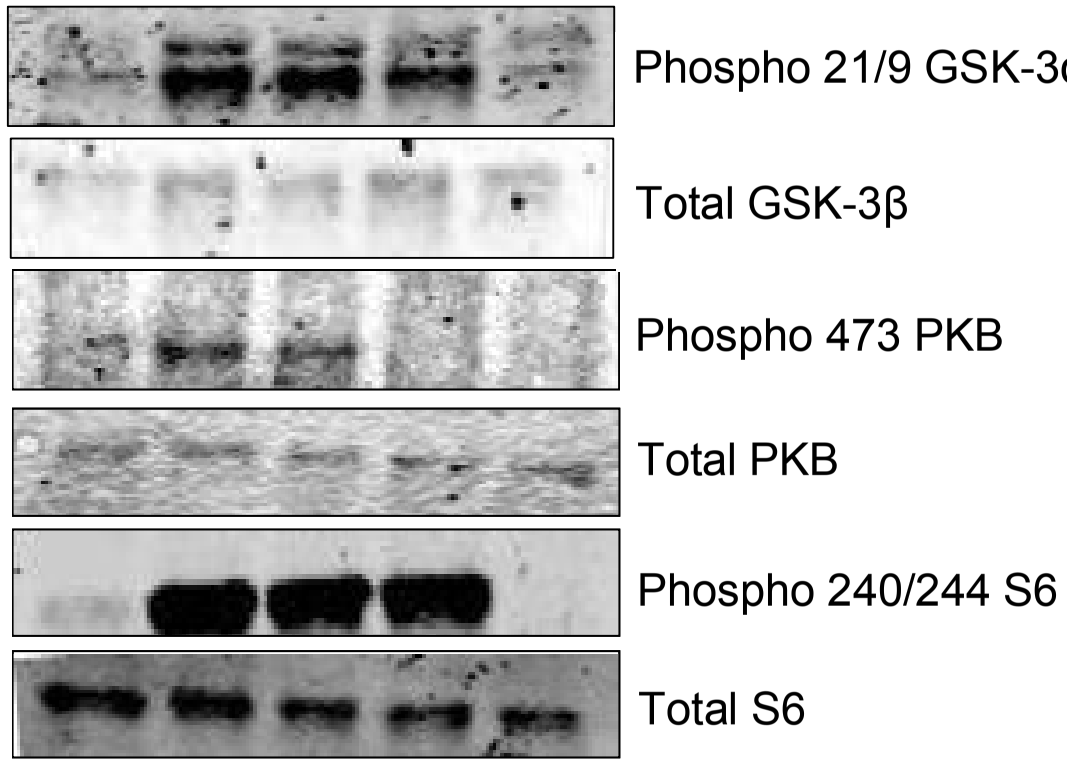


Figure 4.23 Effect of inhibitors of GSK-3, PKB and PI 3-K on intracellular signalling pathways in H4IIe cells. Cells were incubated for 30 minutes in the presence of inhibitors as indicated prior to treatment with insulin for 1 hour before lysis and analysis by western blotting using the antibodies shown.

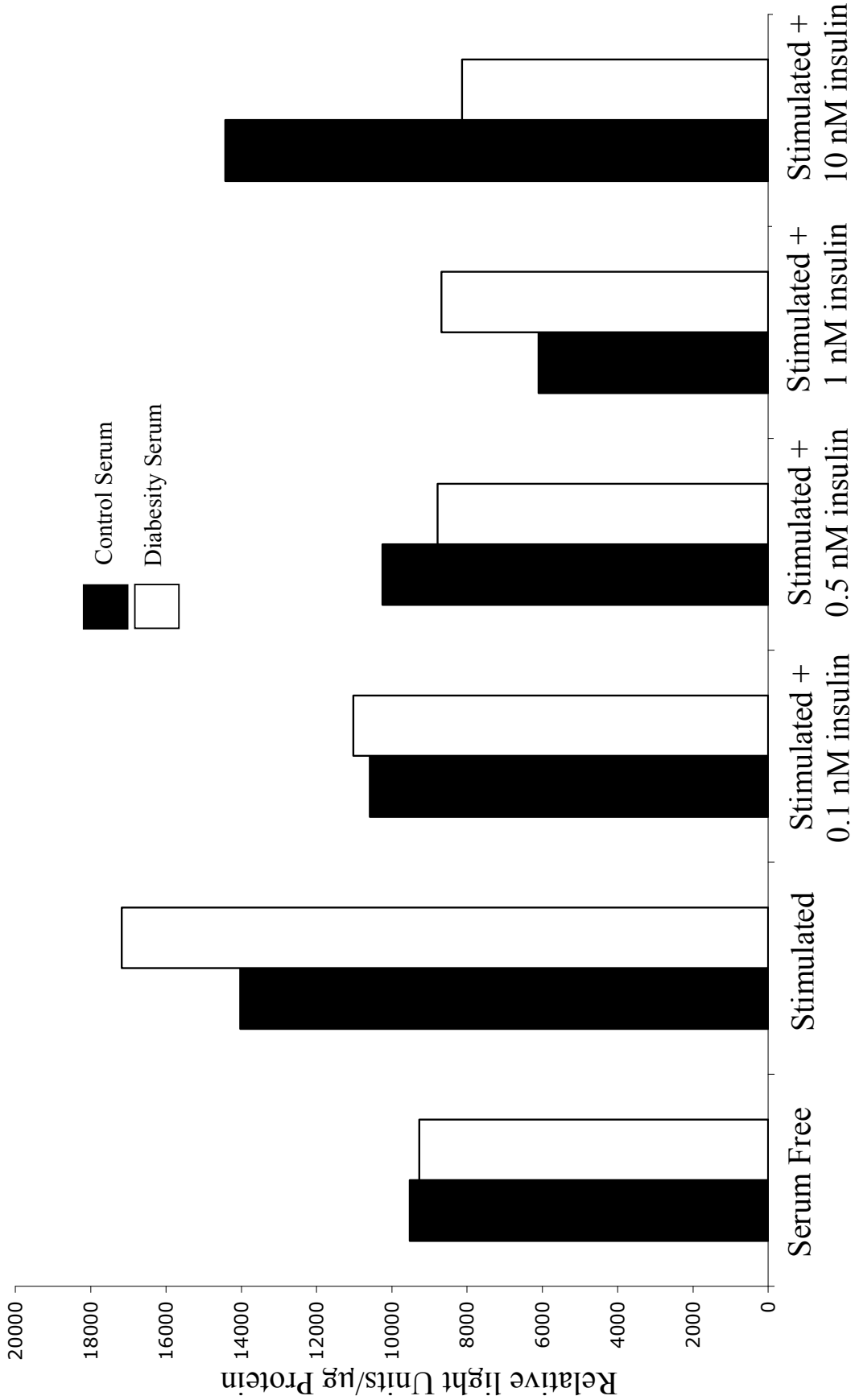


Figure 4.24 Reporter cell lines were ineffective for the purpose of measuring insulin resistance in this cell model. LLRP7 cells were cultured for 3 weeks in DMEM + 5% serum from either a control or diabetic volunteer. After 3 week, cells were fasted for 3 hours prior to 16 hours treatment as indicated. n=1.

freezer failure that caused the loss of the patient serum samples I was not able to repeat this important experiment.

4.4. Discussion

The major reason for developing these reporter cell lines was to increase the speed, ease and cost of the assay to

i) permit development of a cell based screen,

ii) aid the identification of the factor in diabesity serum responsible for insulin resistance; and

iii) aid the analysis of the signalling pathways connecting the insulin receptor to each gene promoter

The first step in achieving these 3 goals was to generate H4IIe cells stably transfected with constructs which produce luciferase in response to activity of the gene promoters of rat PEPCK, human PEPCK, human IGFBP-1 or human G6Pase. In this chapter I have described the production of these cells and the optimisation involved to establish and isolate the most appropriate lines and conditions to monitor reporter regulation. Clearly further investigation of these clones is required to be sure that they are really surrogate reporters of the endogenous gene promoters.

4.4.1. Signalling analysis

Previous work has established the importance of PI 3-K in the regulation of all of these gene promoters (Sutherland et al., 1995, Dickens et al., 1998, Band and Posner, 1997). The effect of PI 3-K inhibitors on reporter cells showed that this part of the insulin signalling pathway is intact in LLRP7, CSHP12 and LLHG4 cells. Activation of PKB is also required for insulin signalling to PEPCK, G6Pase and IGFBP-1 (Logie et al., 2007, Cichy et al., 1998). Inhibition of PKB had partial effects on insulin regulation of each of the reporter lines. Downstream of PKB there is evidence that inhibition of GSK-3 regulates the PEPCK, IGFBP1 and G6Pase gene promoters (Lochhead et al., 2001,

Finlay et al., 2004) . In my reporter cells, GSK-3 inhibition only had a minor effect on LLRP7 cells, and little effect on G6Pase or IGFBP1 reporter lines. It is not clear whether this means that the GSK-3 responsive element lies outwith the promoter regions cloned, whether the GSK-3 effect is primarily on mRNA stability rather than transcription, or it is related to the different time course of the experimental protocol (3 hours for mRNA and 16 hours for luciferase), where the compound may not be stable for longer periods or only affect initial repression of the gene. Endogenous IGFBP-1 differs from the other genes insomuch as insulin regulation requires mTOR (Patel et al., 2002). However rapamycin had no effect on the insulin repression of the IGFBP1 reporter line. Again this may be due to the longer incubation times of this system. Indeed there is evidence that an mTOR independent, PI 3-kinase dependent pathway becomes more important in the regulation of IGFBP-1 in longer incubations with insulin (Finlay et al., 2006).

There is a difference in the response of rat and human PEPCK promoters to both inhibitors and in the response to insulin. For example, insulin stimulates the expression of luciferase in LLRP7 cells, but represses this in CSHP12 cells. The response to PI 3-K and PKB inhibition is similar, but inhibition of GSK-3 blocks the effects of insulin on the repression of luciferase activity in LLRP7 cells only. There are a number of possibilities for these differences. One of the reasons for creating these cell lines was to humanize the cell model by inserting human gene promoters into rat hepatoma cells. The differences between species may have an effect on signalling between the insulin receptor and the gene. Furthermore, in rats PEPCK is almost exclusively contained in the cytoplasm in contrast to humans where 50% of PEPCK is localized to the mitochondria (PEPCK-M). The two isoforms each have their own gene promoters. It is possible that there are parts of the gene promoter 5’ to that which has been cloned which

have effects on gene expression, and therefore luciferase activity. Because they are not present the size of the inserts may also have effects on the response to insulin and inhibitors. Finally, the site of insertion of the construct into the genome may also affect the expression of luciferase. The inherent activity, and any response elements in the area surrounding the gene promoter, could interfere with the signalling pathways that would normally stimulate or repress gene expression.

4.4.2. Temporal effects of measuring luciferase rather than mRNA:

The rationale for developing reporter cell lines was to increase the speed of analysis and for use as a cell based model of diabetes. It takes 16 hours for adequate stimulation of the reporter in all lines. This is despite the actions of dexamethasone and cAMP on the gene promoter occurring within 30 minutes and insulin being even faster (Duong et al., 2002). This lag is likely due to the time it takes for translation of the luciferase protein. This length of time may limit the usefulness of the assay. The level of insulin resistance seen at mRNA level is not a complete abrogation of insulin action, but a shift in insulin sensitivity. It is, as yet, unknown how long it would take for insulin resistance to be reversed in the cell model. The reporter cell will be devoid of the insulin resistance generating serum for 19 hours at the time of analysis and this length of time may be enough to reverse any insulin resistance generated by exposure to diabesity sera.

The cell model was designed to assess the transcriptional activity of each gene promoter. It takes 16 hours of stimulation with dexamethasone and cAMP for the most robust increase in luciferase activity. However, the protein produced is incredibly stable. This protein stability means that any that is produced remains in the cells and as such is not a direct reflection of only transcriptional activity. It may have been better to include a DEAD box within the protein sequence. These domains can interact with mRNA to

promote degradation more rapidly (Py et al., 1996). It also possible to use a luciferase vector containing protein destabilisation sequences such as hCL1 and hPEST (Li et al., 1998, Gilon et al., 1998). These allow a more rapid turnover of luciferase protein making the reporter more sensitive to changes in transcription. If these approaches had been used, the luciferase activity may have been more responsive to hormonal manipulation, both stimulatory and repressive, and as such the readout may have more accurately represented the transcriptional activity of the gene promoters.

4.4.3. Site of Insertion and pGL4.17

The transfection process leads to integration of an unknown number of copies of the plasmid into random regions of the genome. The activity of the integrated DNA will be affected by the background activity of the surrounding genome, added to this, one needs to periodically maintain selection pressure to ensure that the transgene is not shed from the genome. To overcome this, several clones were investigated to ensure that the gene of interest was being studied rather than the flanking sequence. If a large number of copies of the gene are present, the response to hormones may swamp the effects of insulin resistance seen in the endogenous gene promoter of which there is one copy. I did attempt to make isogenic clones using the FLP-In system (Invitrogen), however this was ultimately unsuccessful.

In addition insulin appears to acutely stimulate luciferase activity in many of the clones in the absence of dex and cAMP. This implies that the gene promoter-vector construct contains an enhancer element which responds to insulin. Interestingly this was not the case in every cell line suggesting that it is context dependent, influenced by flanking sequence. In these cases this confounds the repressive effects of insulin on glucocorticoid and cAMP induction of the reporters, resulting in weaker apparent

reduction of reporter. In most cases this could be minimised by using an insulin concentration more optimal for repression than induction, however it still complicates the study of insulin signalling and the use of the reporter in cell based screens for insulin sensitisers. This may underlie some of the distinct effects of signalling inhibitors seen in these cells compared to monitoring endogenous gene transcription (e.g. GSK-3 inhibitor studies). Indeed we also do not know what the generation of insulin resistance would do to the stimulatory effect of insulin on luciferase production. It is also possible that higher dose insulin is acting via the IGF-1 receptor leading to an increase in luciferase production, although this would not explain why the anticipated effect was only seen in three of the four cell lines.

4.5. Conclusions

I have developed and tested four reporter cell lines. This has increased the speed and ease of assay of transcriptional activity of insulin responsive genes. There appear to be some differences in the signalling pathways from the insulin receptor to the reporter genes compared to those regulating the endogenous genes, and these may be related to the presence of an insulin enhancer element in the vector used to generate the cells. This may limit the use of the cell model for the study of insulin signalling. In the next chapter I obtain further evidence that the reporter lines may not be useful as an insulin resistant cell model for the identification of insulin sensitising agents.

Chapter 5. Assessment of Post-Receptor Signalling in Insulin Resistant Cells

5.1. Introduction

Insulin resistance, and relative insulin deficiency, is evident in type 2 diabetes. The underlying molecular mechanisms for the development of this resistance are as yet unknown. The milieu of serum components known to influence whole body insulin sensitivity includes endocrine factors (insulin, glucagon and adipokines), inflammatory mediators (IL-6 and TNFα) and nutrients (glucose and free fatty acids). The levels of these factors are all altered with obesity and in individuals with diabetes. However, before the clinical presentation of diabetes, insulin resistance develops along a continuum and the first molecular step and early influences in the generation of this resistance have remained elusive.

There is little evidence for reduced number of insulin receptor on liver, muscle or adipose being the cause of insulin resistance. Therefore it is widely assumed that a post-receptor signalling defect underlies the loss of tissue sensitivity to this hormone and subsequent clinical symptoms (see Chapter 1.7 for review). Identifying the exact location of the initial signalling problem leading to insulin resistance would aid the development of interventions with efficacy at this key stage of disease initiation, prior to the appearance of most of the diabetes associated health problems. The insulin signalling molecules IRS, PI 3-kinase, PKB and GSK-3 have all been implicated in cellular insulin resistance (Caro et al., 1987, Aguirre et al., 2000, Kim et al., 1999b, Cozzone et al., 2008, Nikoulina et al., 2000). However, it has never been elucidated if loss of regulation of any of these kinases is the initial causative problem associated with development of insulin resistance. Furthermore, the development of insulin resistance may involve different disturbances in the signalling pathway in different metabolic tissues. For example, the transcriptional co-activator, PGC1α has opposing actions in

healthy muscle and liver (Liang et al., 2009), hence a single problem with the regulation of this protein would not have the same effect in both tissues.

Culturing H4IIe cells in the serum from individuals with diabesity induces insulin resistance as measured by the reduced ability of insulin to suppress PEPCK gene transcription (Chapter 3.2.2). In traditional models of insulin resistance, high doses of individual compounds, e.g. ceramide, are used to generate insulin resistance and subsequent signalling changes proposed to be key for the generation of resistance in humans (Ch 1.13.2). This focussed approach, although useful, does not really establish the effects of a more physiologically relevant mixture of endocrine, inflammatory and nutrient components on insulin signalling pathways. We argue that our unbiased approach is a better starting point to investigate signalling defects in response to the real disease. Subsequently, normal serum could be fortified with, or diabesity serum depleted of, many of the proposed individual factors associated with insulin resistance. This would assess the requirement and sufficiency of each factor to generate the same defects on insulin signalling seen with complete diabesity serum.

Hyperglycaemia, although resistance generating, is the final step in the development of type 2 diabetes and it is therefore unlikely that this is the initial direct cause of insulin resistance. Also, the level of TNF-α in human serum (Chapter 3.2.1) was no different between cases or controls, so this rules it out as the cause of the different effects of the two sera. One of the largest differences between the control and diabesity sera was the insulin levels (Table 3.1). Hyperinsulinaemia is present prior to the development of diabetes, potentially as a normal mechanism to overcome the developing insulin resistance but it is also possibly due to defective insulin secretion or turnover in

response to dietary influences (e.g. obesity), and as such may be one of the first factors on the road to development of tissue insulin resistance.

In this chapter I supplement cell culture medium with insulin to generate the same levels observed in the diabesity serum. H4IIe cells grown in this medium for a prolonged period developed insulin resistance, measured by regulation of the PEPCK gene promoter. I then investigate whether there are any significant changes in post-receptor signalling which could explain the effect on regulation of PEPCK. Finally, I alter post-receptor signalling pharmacologically in an attempt to determine the level of signalling defect that would be required to alter regulation of PEPCK gene transcription.

5.2. Results

5.2.1. Development of insulin resistance in H4IIe cells by prolonged culture in low level supplemented insulin

Foetal calf serum was supplemented with 100 pM insulin and frozen at -20 ˚C overnight. The following morning, the serum was thawed and diluted to 5% in DMEM leaving the total concentration of supplemented insulin in media at 5 pM. H4IIe cells were then cultured for 3 weeks in either media with standard FCS (Standard Media containing a final concentration of 3.8 pM insulin) or FCS supplemented with insulin (Supplemented Media, final insulin concentration 8.8 pM insulin). Cells were passaged twice weekly and each time the media was refreshed. After 3 weeks cells were plated, fasted for 3 hours prior to stimulation with dexamethasone (500 nM) and cAMP (100 μM 8-CPTcAMP) in the presence or absence of increasing concentrations of insulin (0.1 to 10nM). Total cellular RNA was extracted and cDNA synthesised before assessment of PEPCK and actin levels by Taqman analysis. In both groups of cells dexamethasone and cAMP stimulated the production of PEPCK and this was dominantly repressed by insulin. Cells cultured in supplemented media exhibited insulin resistance relative to cells grown in standard media as shown by the reduced ability of 0.1 ($p=0.03$) and 0.5 nM ($p=0.01$) insulin to repress PEPCK from maximal stimulation (Figure 5.1). There was no difference in ether the basal or stimulated levels of PEPCK (Figure 5.1). However, the mean repression by insulin reduced from 48.2 to 5.0% and from 105.6 to 75.8% with 0.1 nM and 0.5 nM insulin respectively in cells exposed to supplemented serum (Figure 5.2). There was no difference between cells grown in control and supplemented medium in the level of PEPCK repression with 1 and 10 nM insulin (Figure 5.2).

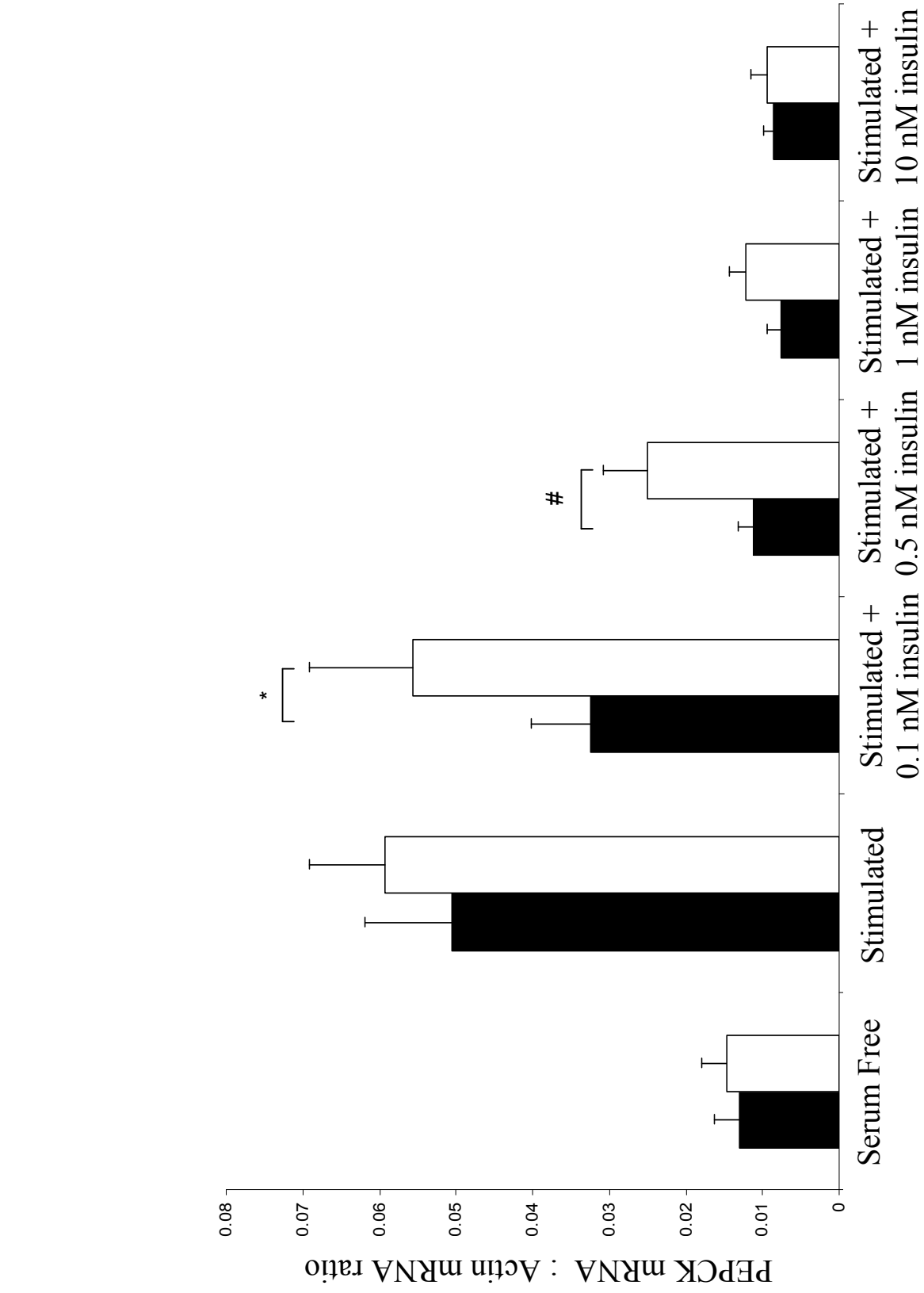


Figure 5.1 Effect of culturing H4IIe cells in enhanced levels of insulin. Cells were cultured for 3 weeks in standard media, or media supplemented with 5 pM insulin. Cells were starved for 3 hours and then treated for 3 hours with dexamethasone and cAMP (stimulated) in the presence or absence of insulin as indicated. Black bars = Standard FCS, White bars = FCS + 5 pM insulin. * = p = 0.03, # = p = 0.01 (n=5 performed in duplicate)

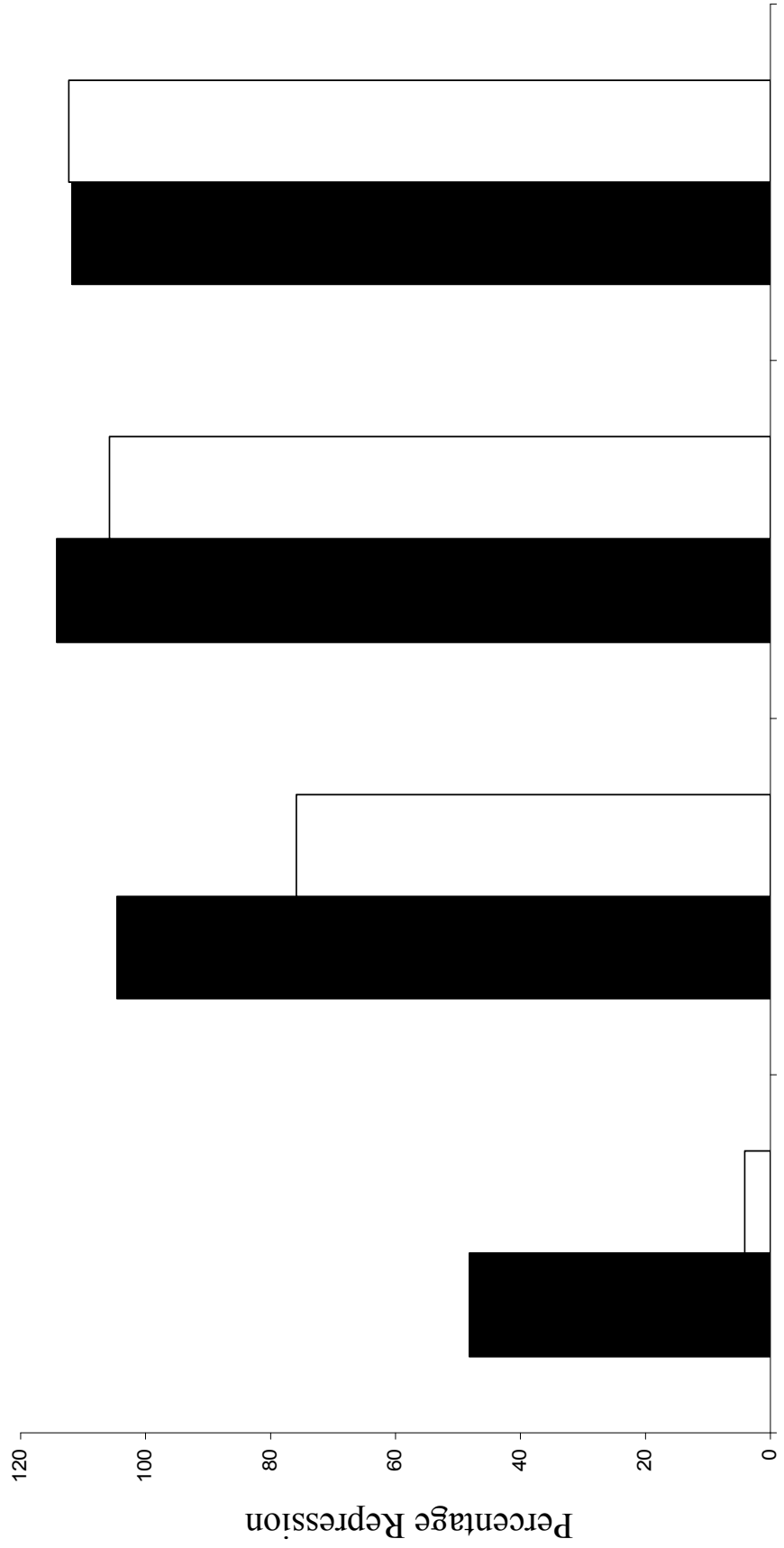


Figure 5.2 Percentage repression of by insulin in H4IIe cells cultured in either standard FCS or FCS supplemented with 5 pM insulin. Black bars = FCS, white bars = FCS + 5 pM insulin

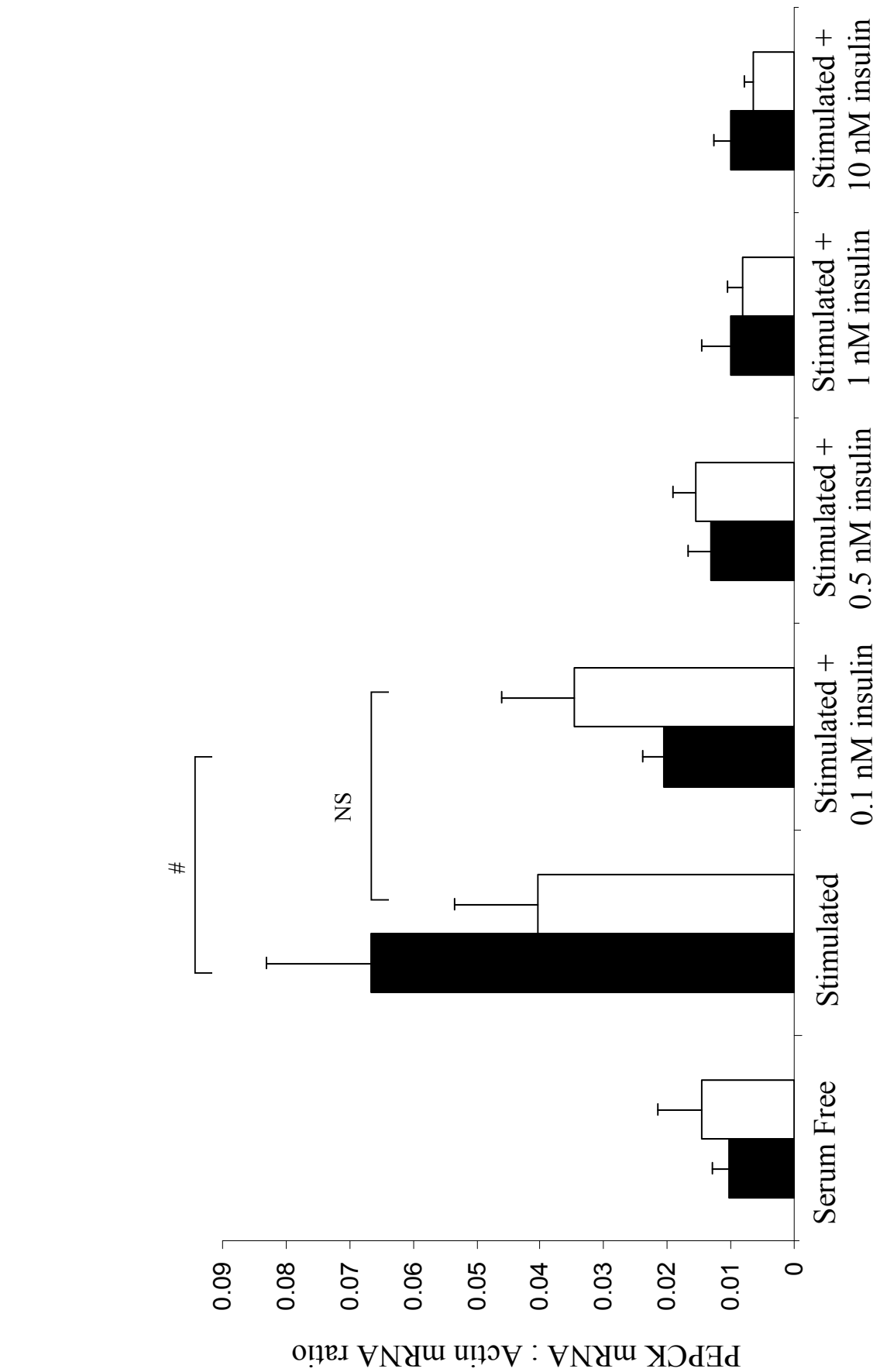


Figure 5.3 H4IIe cells require 3 weeks to develop insulin resistance. Cells were cultured for 2 weeks in standard media, or media supplemented with of 5 pM insulin. Cells were starved for 3 hours and then treated for 3 hours with dexamethasone and cAMP in the presence or absence of insulin as indicated. Samples were analysed by TAQMAN. Black bars = Standard FCS, White bars = FCS + 5 pM insulin (n=3 performed in duplicate). P values, NS=non-significant, # = 0.02

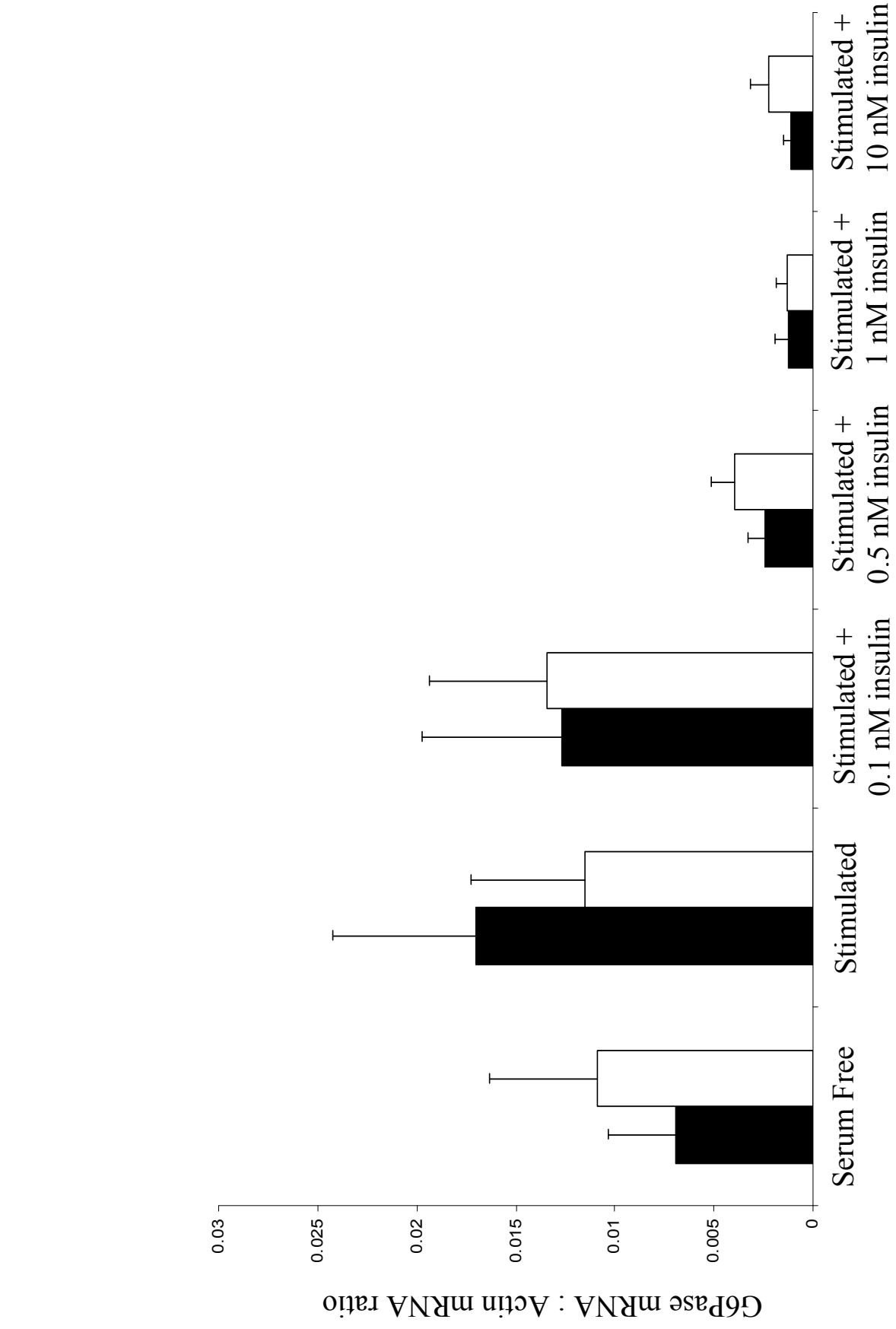


Figure 5.4 Effect of culturing H4IIe cells in enhanced insulin on expression of G6Pase. H4IIe cells were cultured for 3 weeks in standard media, or media supplemented with 5 pM insulin. Cells were starved for 3 hours and then treated for 3 hours with dexamethasone and cAMP in the presence or absence of insulin as indicated. Samples were analysed by TAQMAN. Black bars = Standard FCS, White bars = FCS + 5 pM insulin (n=3 performed in duplicate)

After culture of cells for 2 weeks in supplemented media there was no significant difference in the sensitivity of the PEPCK gene promoter to any concentration of insulin from that found in the control cells (Figure 5.3). However, there was a greater stimulation in cells cultured in standard serum which was repressed bu 0.1 nM insulin. Whereas cells cultured in supplemented serum were stimulated less well but were also not repressed by 0.1 nM insulin. Hence, 3 weeks of exposure to 5 pM insulin was usedd for the development of insulin resistance in the H4IIe cells as the results obtained were more consistent and the effects on PEPCK stimulation were similar

Interestingly, in cells grown in supplemented media for 3 weeks there was no loss of insulin sensitivity of the G6Pase gene promoter (Figure 5.4). It should be noted that the assessment of G6Pase gene transcription was much more variable than that of PEPCK. Nevertheless, it appears that supplemented media has differential effects on the insulin sensitivity of these two genes involved in gluconeogenesis. In standard H4IIe cells, the EC50 for insulin repression of PEPCK and G6Pase is similar, 0.2 nM and 0.16 nM, respectively. Hence the lack of response on insulin regulation of G6Pase is not due to differential sensitivities of these gene promoters for insulin.

5.2.2. The effect of culturing in human serum supplemented with low level insulin

To ensure that this effect was not specific to supplementation of FCS, I next supplemented a human control serum with insulin. Control sample 15, which contained the closest level of insulin to the mean of the controls, (39.6 pM compared to 38.9 pM) was used. The final insulin concentration in cell culture control media was therefore 1.98 pM after dilution to 5% (v/v) serum. For supplemented serum the control serum was adjusted to 100 pM insulin by the addition of 1 uL of 60 nM human insulin to each

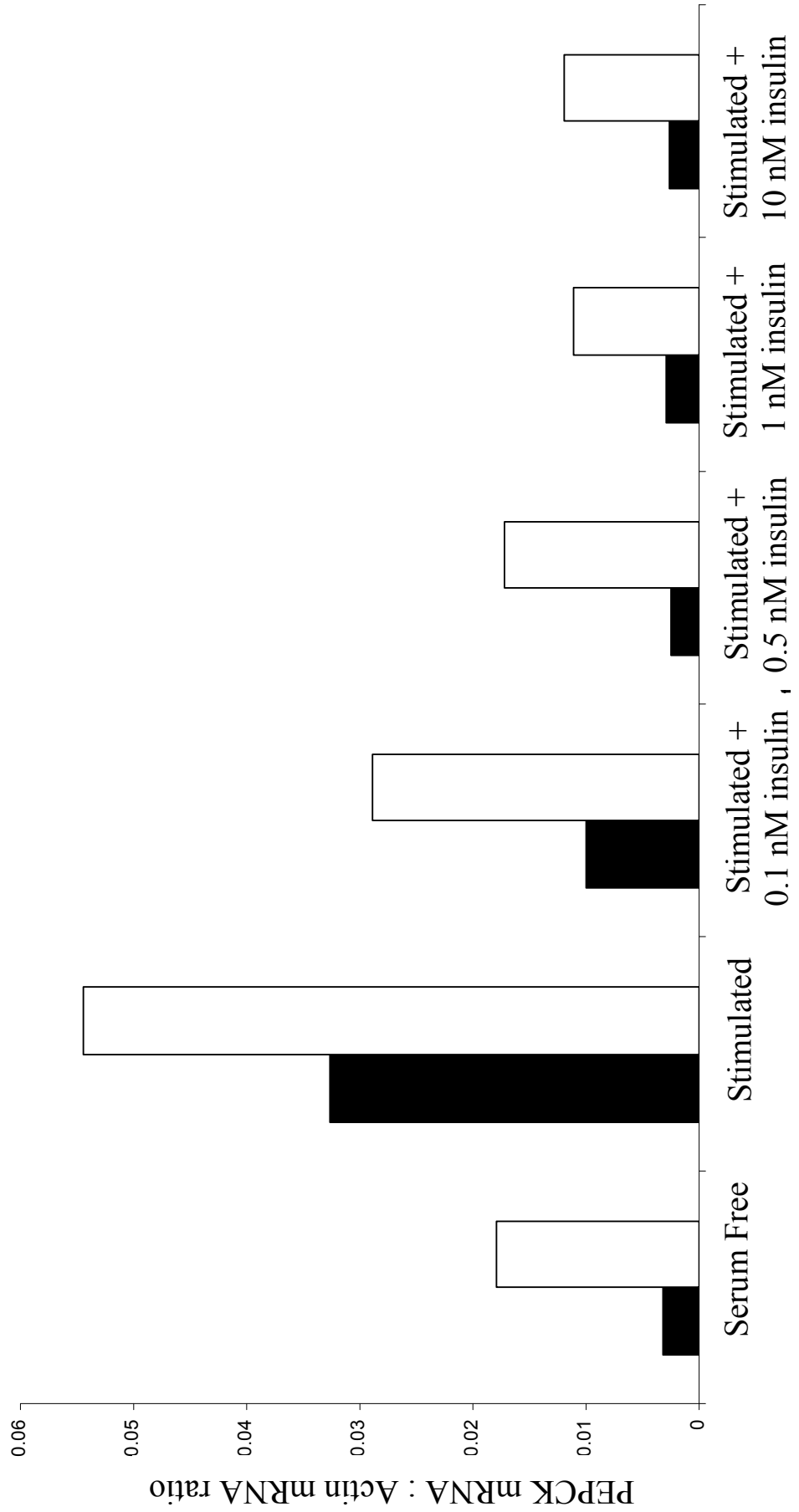
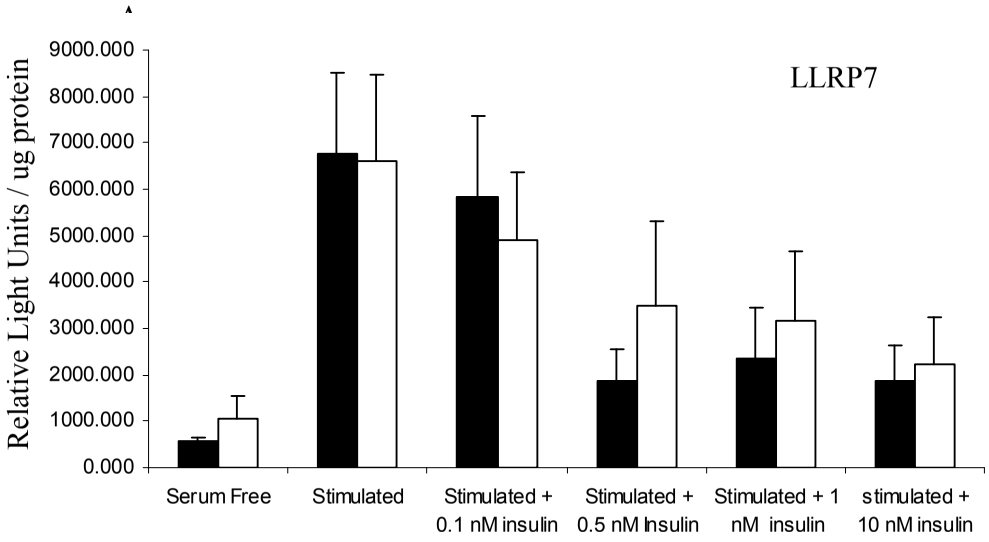


Figure 5.5 Effect of growth in human serum supplemented with 5 pM insulin on PEPCK expression in H4IIe cells. Cells were cultured in serum containing 5% serum from control human 15 with or without the addition of insulin to increase the total concentration to 5 pM. Cells were cultured for 3 weeks, starved for 3 hours and then treated for 3 hours with dexamethasone and cyclic AMP with the addition of indicated levels of insulin. Black bars = control sample 15, white bars = control sample 15 + 5 pM insulin. (n=1)

mL of control serum 15 resulting in an insulin concentration in complete media of 5 pM. H4IIe cells were cultured for 3 weeks in standard or supplemented medium, acutely exposed to dexamethasone and cAMP in the presence or absence of insulin and the mRNA extracted for analysis of PEPCK and actin levels by Taqman. Once again, insulin resistance is evident in cells cultured in supplemented media as shown by a reduction in the ability of both 0.5 and 1 nM insulin to suppress PEPCK (Figure 5.5).

5.2.3. Insulin resistance is not evident in reporter cells cultured in media supplemented with low level insulin

Reporter cells (Chapter 4) were cultured in either standard or supplemented serum as above. LLRP7, CSHP12 and LLHG4 cells were cultured for 3 weeks in each media. The cells were then fasted for 3 hours followed by treatment for 16 hours with dexamethasone and cAMP in the presence or absence of varying concentrations of insulin. Cells underwent lysis and assessment of luciferase activity. The protein concentration of each sample was assayed and luciferase activity corrected for protein level. Insulin resistance was not evident in any of the reporter cells (Figure 5.6). There is no real difference in either the basal, stimulated or insulin treated levels of luciferase in either LLRP7 and LLHG4 cells cultured in supplemented serum and CSHP12 cells have a lower level of stimulation under these conditions (Figure 5.6). Hence, when expressed as percentage repression by insulin there is no significant effect of culturing in supplemented medium compared to standard medium. This is similar to the effects witnessed when culturing reporter cells in control and diabesity serum as discussed in chapter 4.



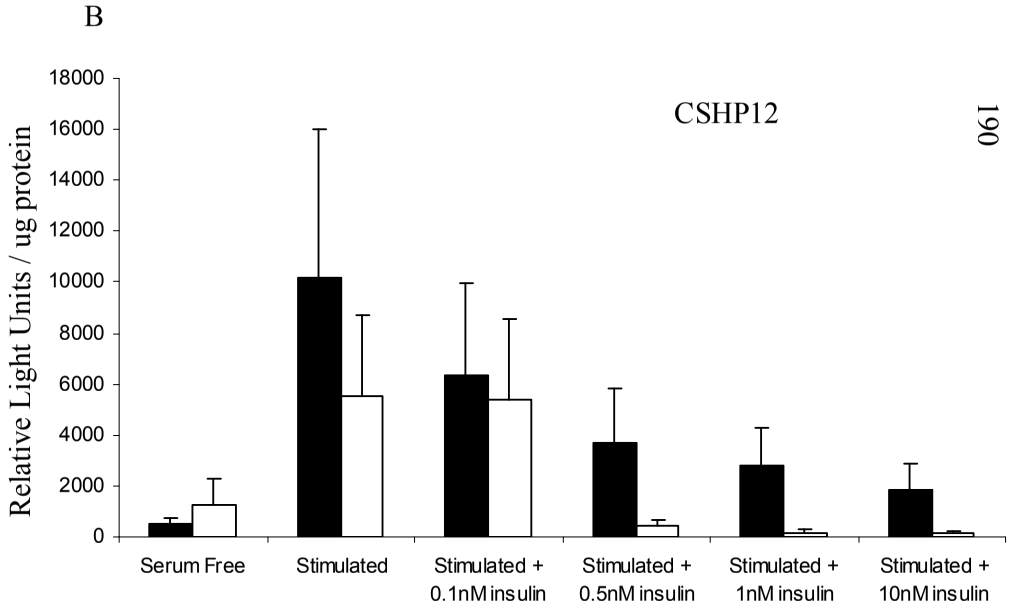


Figure 5.6. LLRP7 (n=4) (A), CSHP12 (n=2) (B) and LLHG4 (n=3) (C) cells were cultured for 3 weeks in DMEM + 5% FSC +/- 5 pM insulin. After 3 week, cells were fasted for 3 hours prior to 16 hours treatment as indicated. Black bars = Standard Media, White Bars = Standard media + 5 pM Insulin)

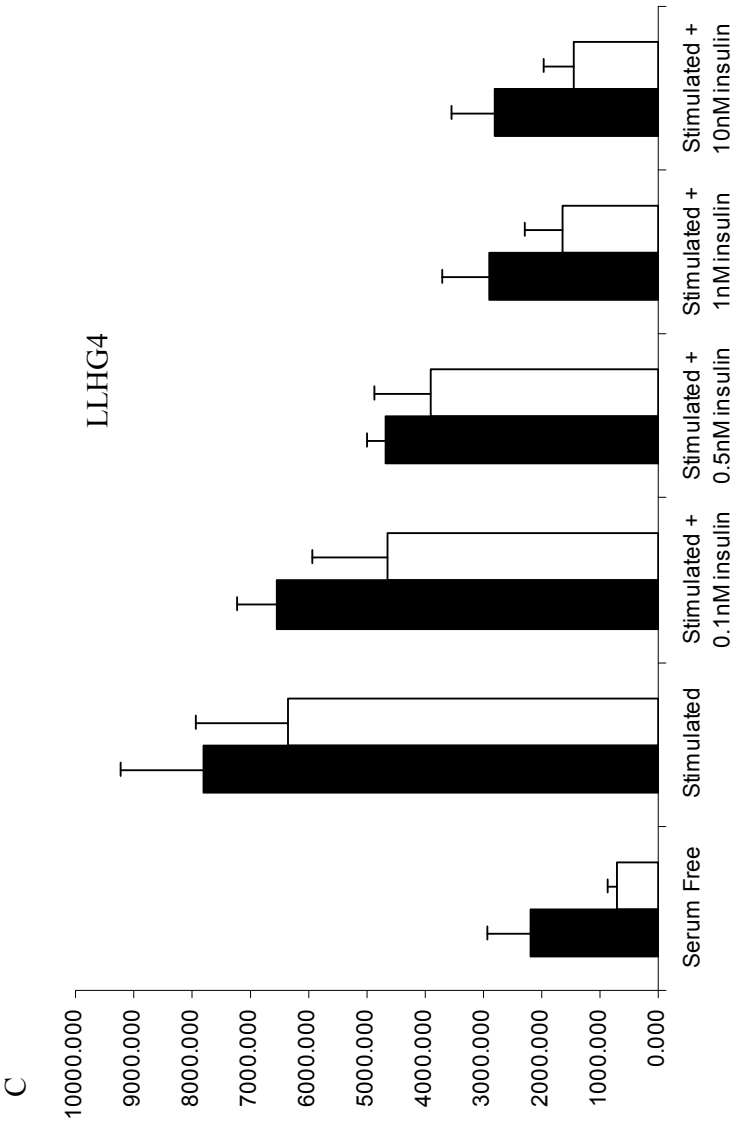


Figure 5.6 continued

5.2.4. Insulin signalling in insulin resistant cells

I next investigated the insulin sensitivity of post-receptor signalling in the H4IIe cells (generated in 5.2.2) in order to establish whether loss of insulin regulation of PEPCK after exposure to supplemented medium could be explained by reduced signalling. After 3 weeks incubation in either standard or supplemented serum the cells were fasted for 3 hours followed by treatment for 1 hour with insulin. The cells were then lysed and analysed by Western blotting. There was no difference in the phosphorylation levels of PKB, S6 ribosomal protein or MAPK between cells cultured in standard medium and cells cultured in supplemented medium (Figure 5.7). However, as Western blotting is semi-quantitative it may not be a sensitive enough modality to see any subtle changes in phosphorylation that may influence insulin sensitivity of downstream targets.

During these experiments I noticed that not all insulin signalling pathways were equally sensitive to insulin even in control cells. To investigate more thoroughly I exposed H4IIe cells to insulin ranging from 5 to 10,000 pM and monitored each of the main pathways by Western blotting (Figure 5.8). This showed that individual signalling molecules require different concentrations of insulin for significant changes in phosphorylation (Figure 5.8). There is clearly no induction in the phosphorylation of PKB or p42/44 MAPK by 5 pM insulin (Figure 5.8 A, B and D). However, 5 pM insulin has a small effect on phosphorylation of S6 ribosomal protein (Figure 5.8 A and C). In fact, the concentration of insulin required for 50% maximal phosphorylation of S6 is 65 pM, whereas for p42/44 MAPK and PKB this is 1241 pM and 2738 pM respectively (Figure 5.9).

S6 ribosomal protein kinase has negative regulatory effects on IRS-1 which may in turn impact on insulin signalling (see Chapter 1.6.2 for details), therefore it was possible that

chronic exposure to this low level of insulin (5 pM) could promote weak but chronic S6K activation that could antagonise IRS-1 signalling. However, there was no significant difference in the total levels of IRS-1 between the cells cultured in standard and supplemented insulin (Figure 5.10). I next investigated whether specific phosphorylation of IRS-1 had changed following culture in low level insulin. After culture for 3 weeks in standard or supplemented insulin the cells were fasted for 3 hours and then treated for 15 minutes with 1 nM insulin. The cells were lysed and immunoprecipitation performed with anti-IRS-1 antibody prior to SDS PAGE and probing with antibodies to phospho-Ser307 IRS-1 or phospho-Tyr (4G10). Again, there was no difference in Ser phosphorylation of IRS-1 detectable between cells cultured in standard or supplemented media (Figure 5.11). Similarly, when immunoprecipitation was performed with total IRS-1 antibody and this probed with a phospho-Tyr antibody (4G10), there was no difference between cells cultured in standard media or supplemented media (Figure 5.12). In contrast, when immunoprecipitation was performed with the phospho-Tyr antibody and this probed with total IRS-1 there was less signal from cells cultured in supplemented media compared to those cultured in standard medium ($p=0.03$ Figure 5.13). This was the only indication that there was a deficit in insulin signalling in cells cultured in 5 pM insulin (i.e. reduced Tyr phosphorylation of IRS-1), however it isn't clear why it was only detected in pull downs in one direction (Fig 5.12 vs. 5.13). Further evidence that there is not a significant deficit in IRS-1 induction of PI 3-kinase comes from analysis of the cellular levels of PIP3 (the product of PI 3-kinase activity). H4IIe cells were cultured for 3 weeks in either standard or supplemented media. Cells were then fasted for 16 hours prior to incubation with or without 0.5 and 10 nM insulin for ten minutes prior to lysis. Dr Alex Grey analysed the PIP3 levels in the samples (Chapter 2.2.16), and found no difference

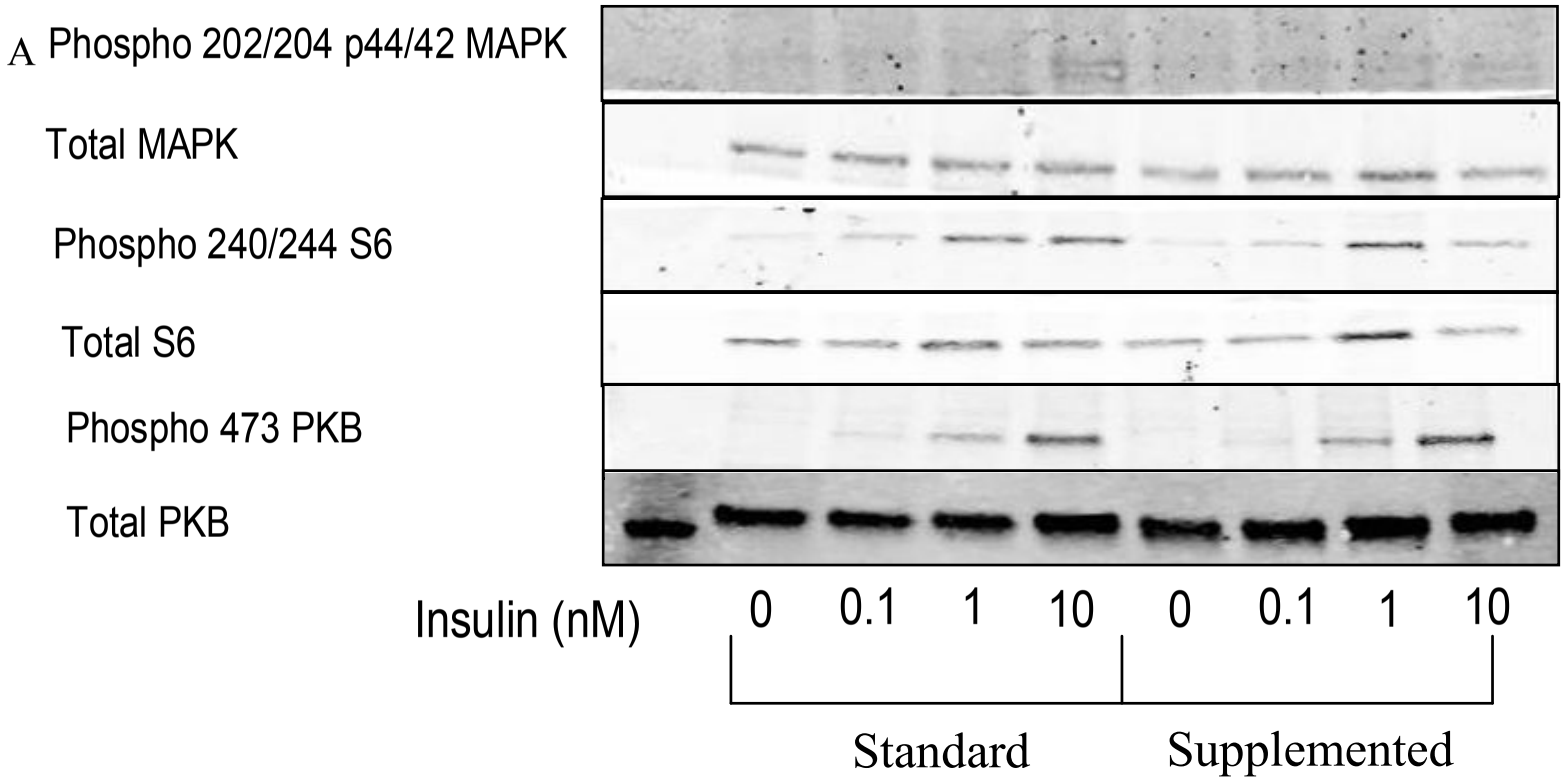


Figure 5.7 Intracellular signalling in H4IIe cells after 3 weeks in different culture conditions. Cells were cultured for 3 weeks in standard media (Standard), or media with the addition of 5 pM insulin (Supplemented). Cells were starved for 3 hours and then treated for 1 hour with insulin as indicated. Protein was extracted and (A) analysed by western blotting using the indicated antibodies. Densitometry of western blotting. (B) PKB, (C) S6 Ribosomal Protein, (D) p42/44 MAPK. Black Bars =Standard Serum, White bars = Supplemented Serum. (n=2)

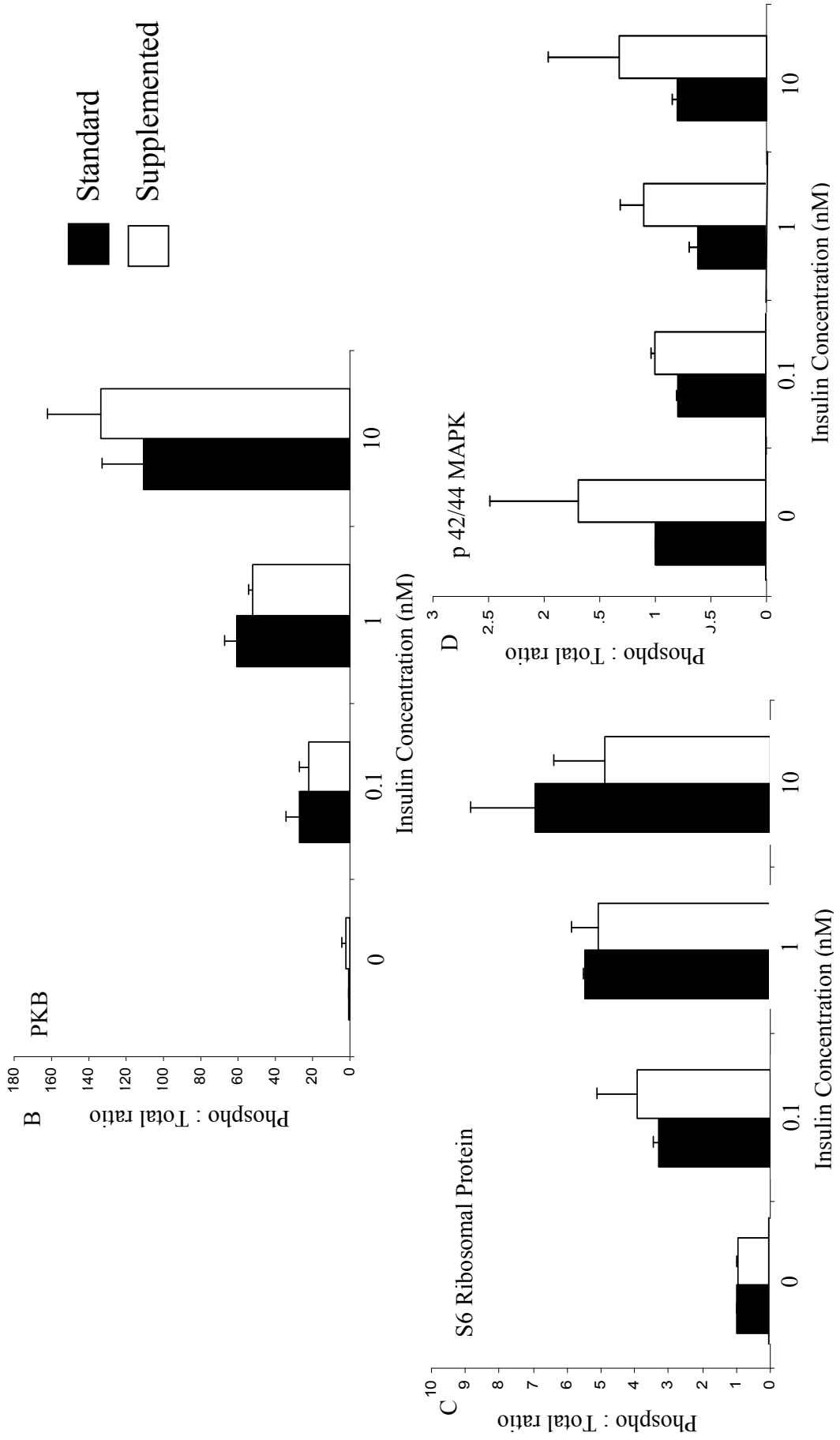


Figure 5.7 Continued

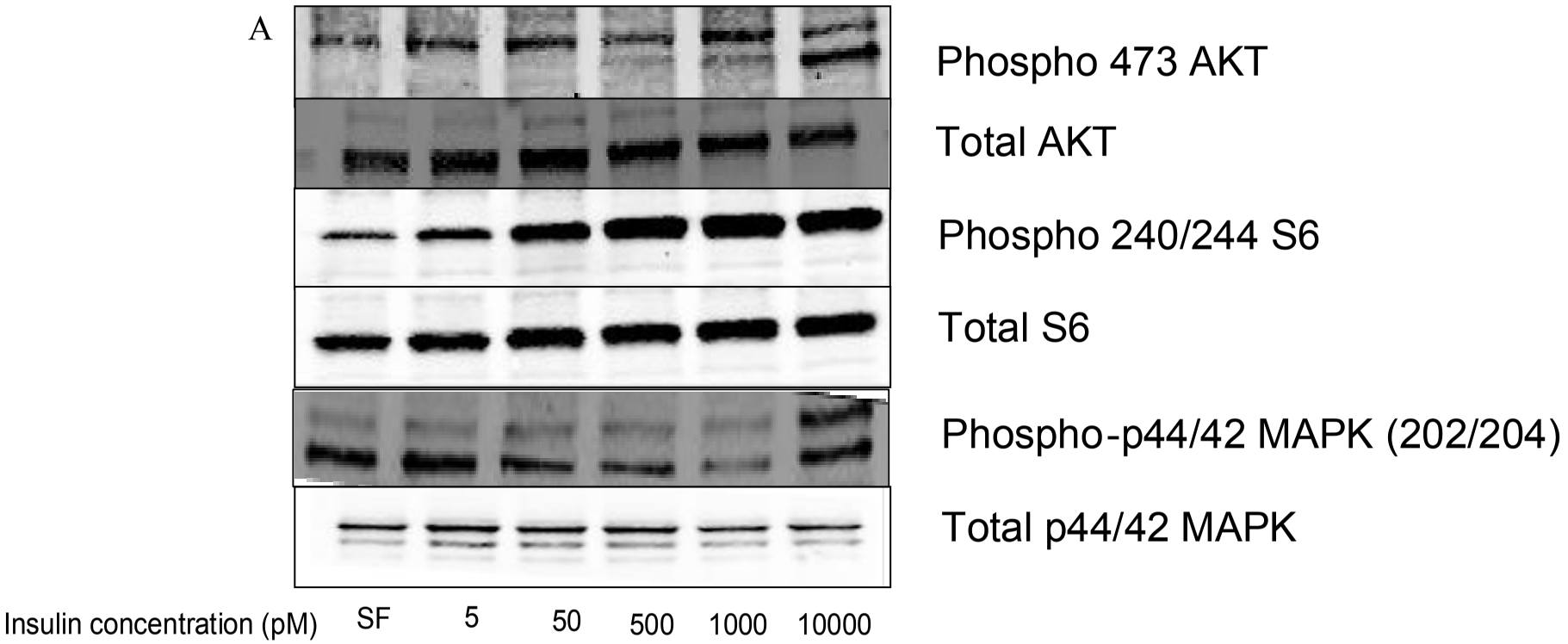
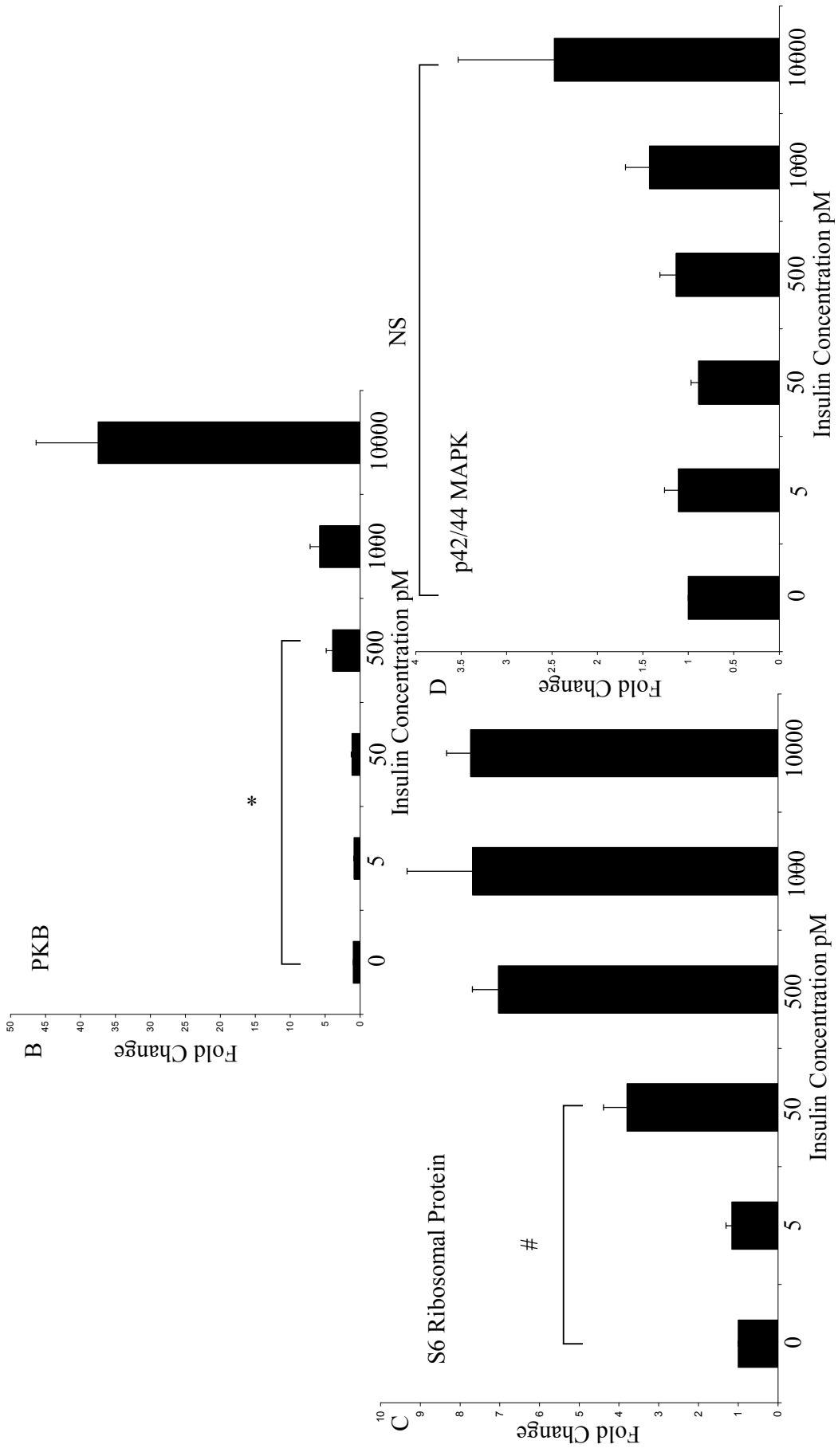


Figure 5.8 Effect of picomolar concentrations of insulin on acute intracellular signalling in H4IIe cells. Cells were fasted for 3 hours and then treated for 1 hour with insulin as indicated. Lysis was performed before proteins were analysed by Western blotting using the indicated antibodies. (A) Representative Blot. (B,C,D) Densitometry of Figure A Data shown as fold change in phospho to total protein ratio with mean and standard error depicted. n=3 (B) Protein Kinase B, (C) S6 ribosomal protein, (D) p42/44 MAPK. (n=3). P values NS = not significant, *= 0.05, #=0.001



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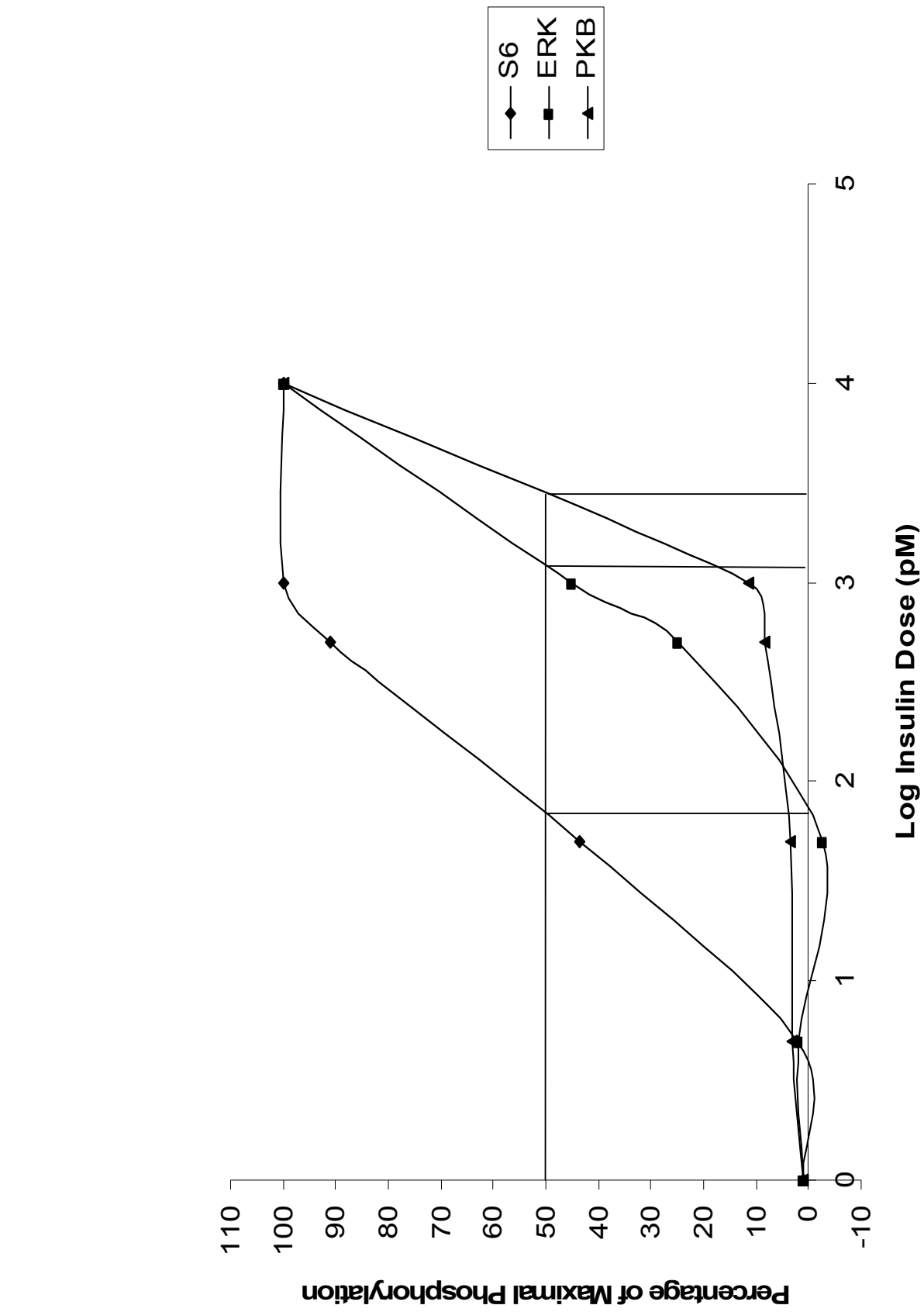


Figure 5.9 Dose response curves for major insulin signalling pathways. Data shown as mean percentage of maximum phosphorylation related to basal. Squares = S6 ribosomal protein Triangles = ERK, Diamonds = PKB. ED 50 of insulin on, S6 =65 pM, ERK = 1241 pM, PKB = 2738 pM. (n=3)

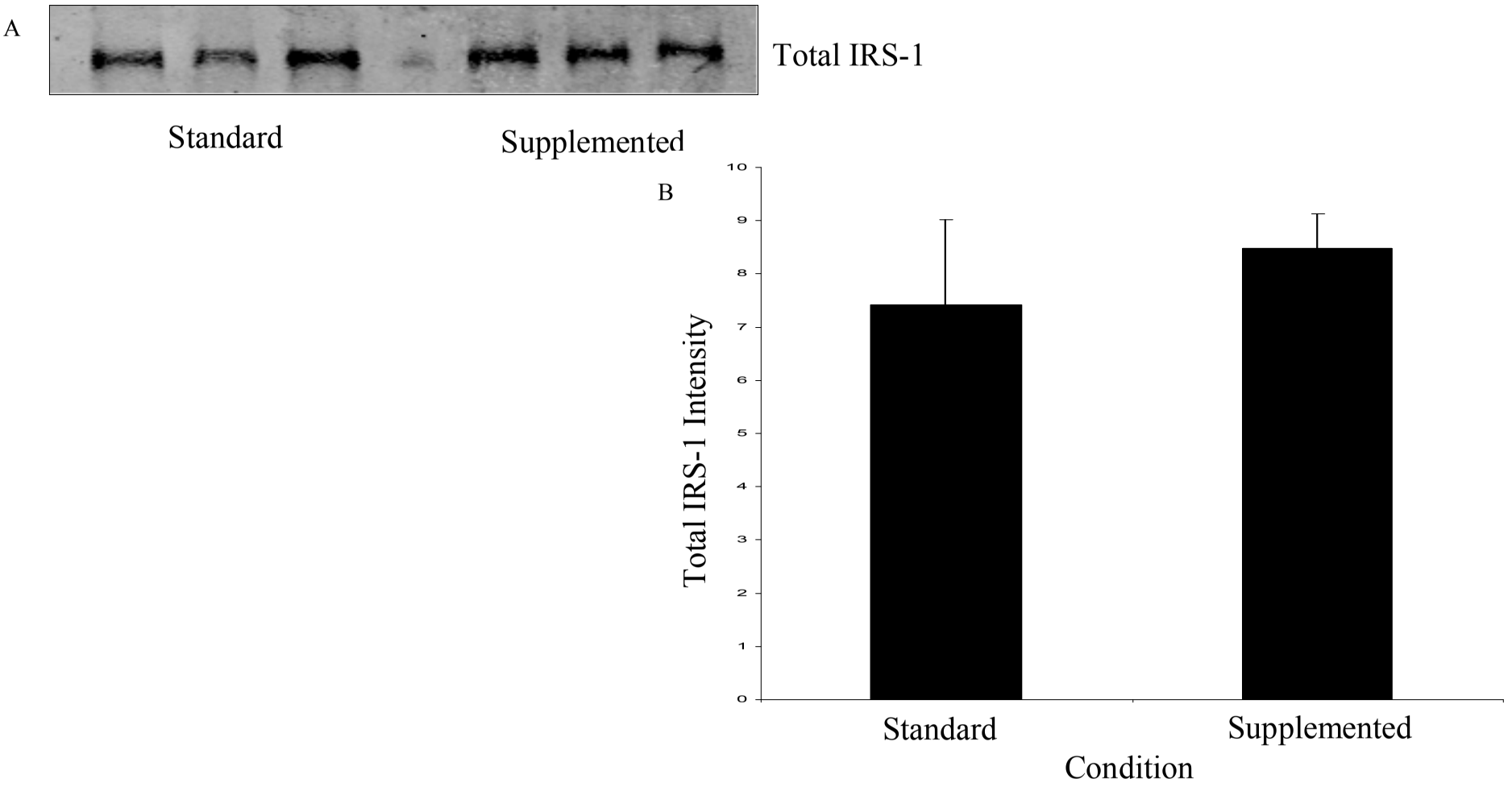


Figure 5.10 Total IRS-1 levels in H4IIe cells cultured for 3 weeks in Media with 5% FCS (Standard) or Media with 5% FCS supplemented with 5 pM insulin (Supplemented). Cells were fasted for 3 hours and then lysed before analysis by western blotting. (A) Representative blot. (B) Densitometry of Total IRS-1 bands. (n=3)

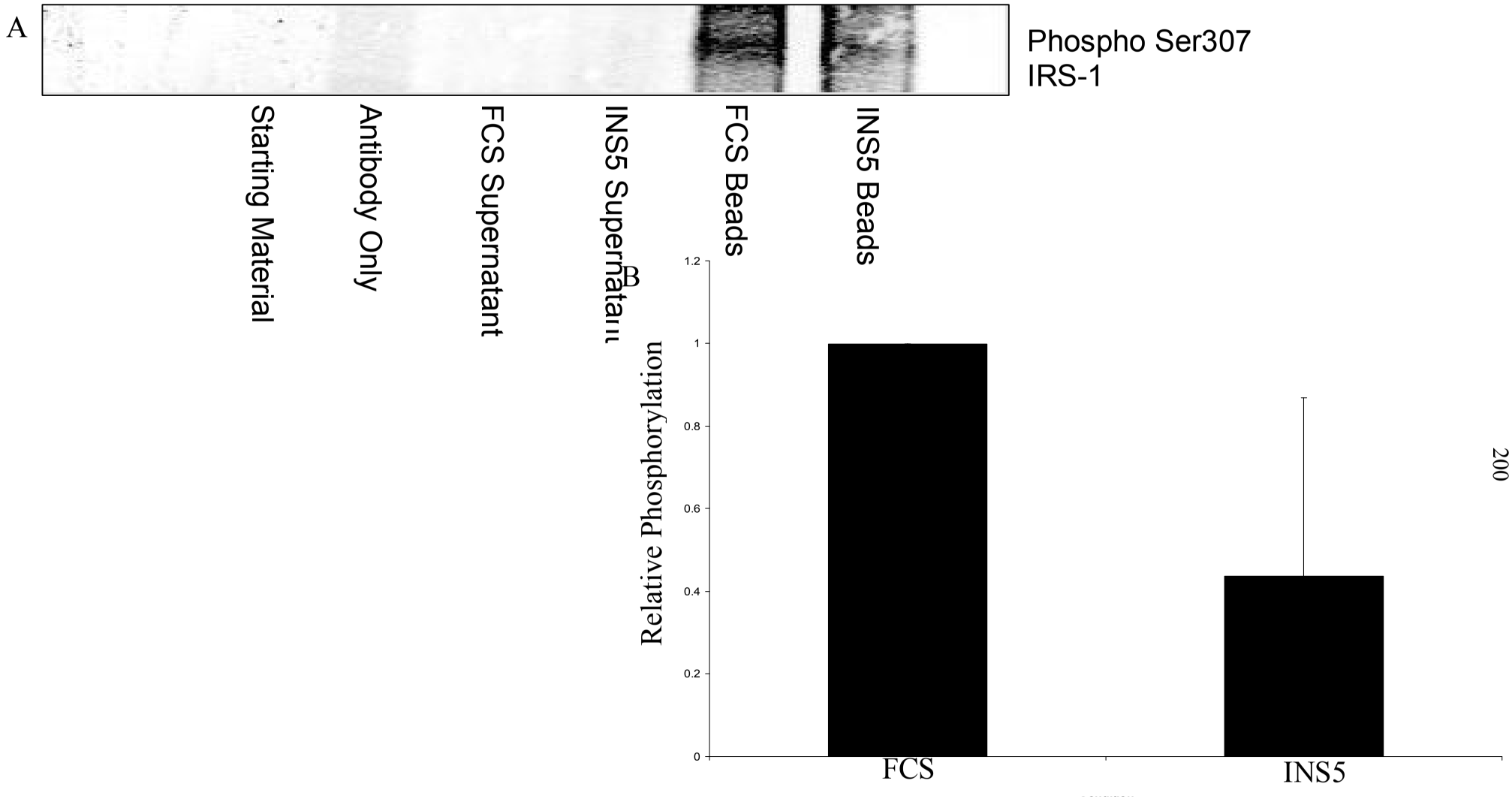


Figure 5.11 IRS-1 Ser phosphorylation. H4IIe cells were cultured for 3 weeks in FCS + (INS5) or (FCS) 5 pM insulin. Cells were fasted for 3 hours then exposed to 1 nM insulin for 15 minutes. IRS-1 was immunoprecipitated from 400 µg of cellular protein with anti IRS-1 antibody before SDS PAGE, then blotted with anti-phospho Ser307 IRS-1 ab. A Representative Blot. B Densitometry shown as relative phosphorylation. Starting material = 30 µL cell lysates, antibody only = 30 µL cell lysates + 1 µg antibody, FCS / INS5 supernatant = 30 µL lysates after incubation with beads + antibody, FCS /INS5 beads = all bead bound protein (n=2)

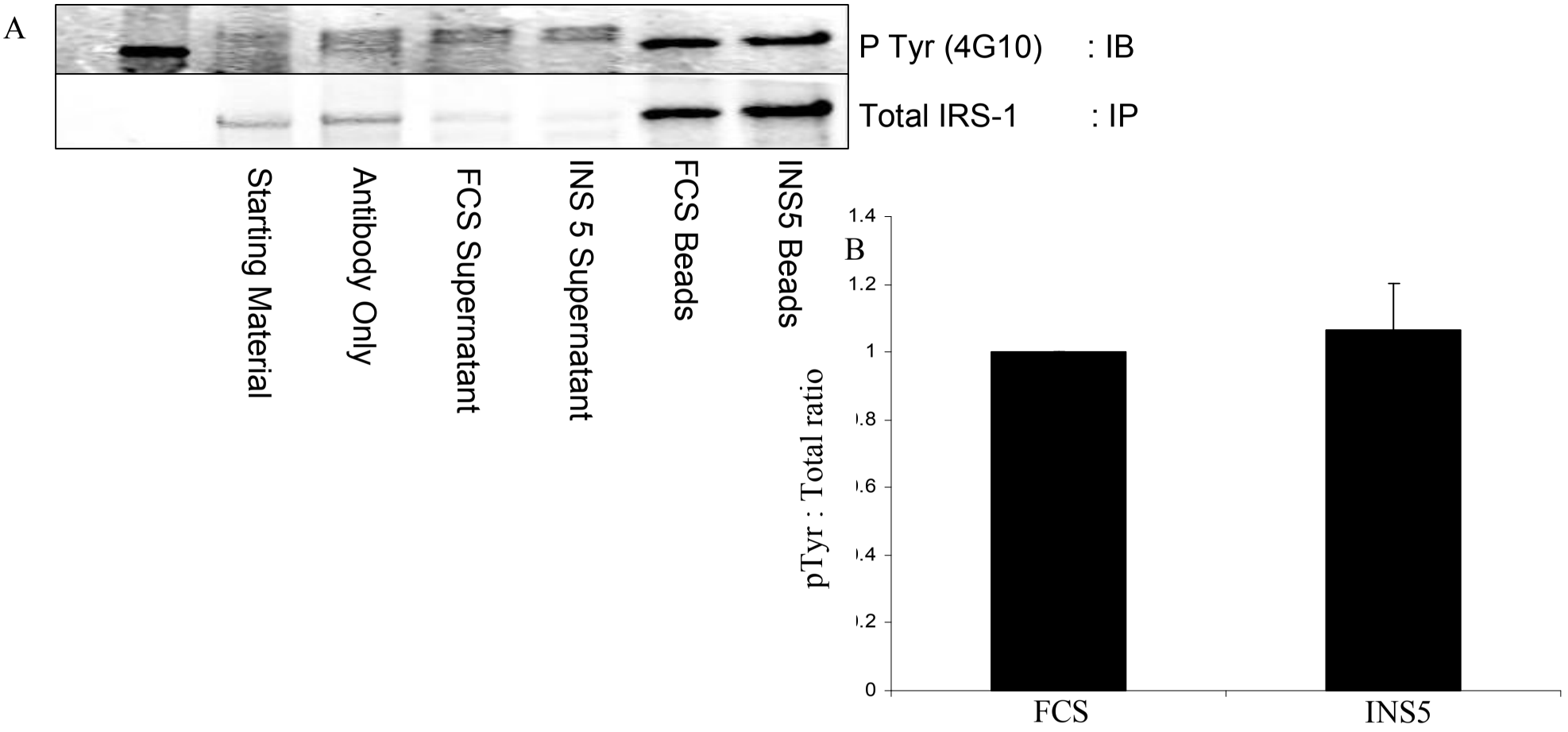


Figure 5.12 IRS-1 Tyr phosphorylation. H4IIe cells were cultured for 3 weeks in FCS + (IR) or - (FCS) 5 pM insulin. Cells were fasted for 3 hours then exposed to 1 nM insulin for 15 minutes. IRS-1 was immunoprecipitated from 400 ug total protein with anti IRS-1 antibody before SDS PAGE then blotted with anti IRS-1 ab or 4G10 anti phosphoTyr antibody. (A) Representative blot of experiment, (B)Densitometry of blots. Data represented as relative phosphorylation of pTyr to total IRS-1. Starting material = 30 µL cell lysates, antibody only = 30 µL cell lysates + 1 µg antibody, FCS / INS5 supernatant = 30 µL lysates after incubation with beads + antibody, FCS /INS5 beads = all bead bound protein.
(n=3)

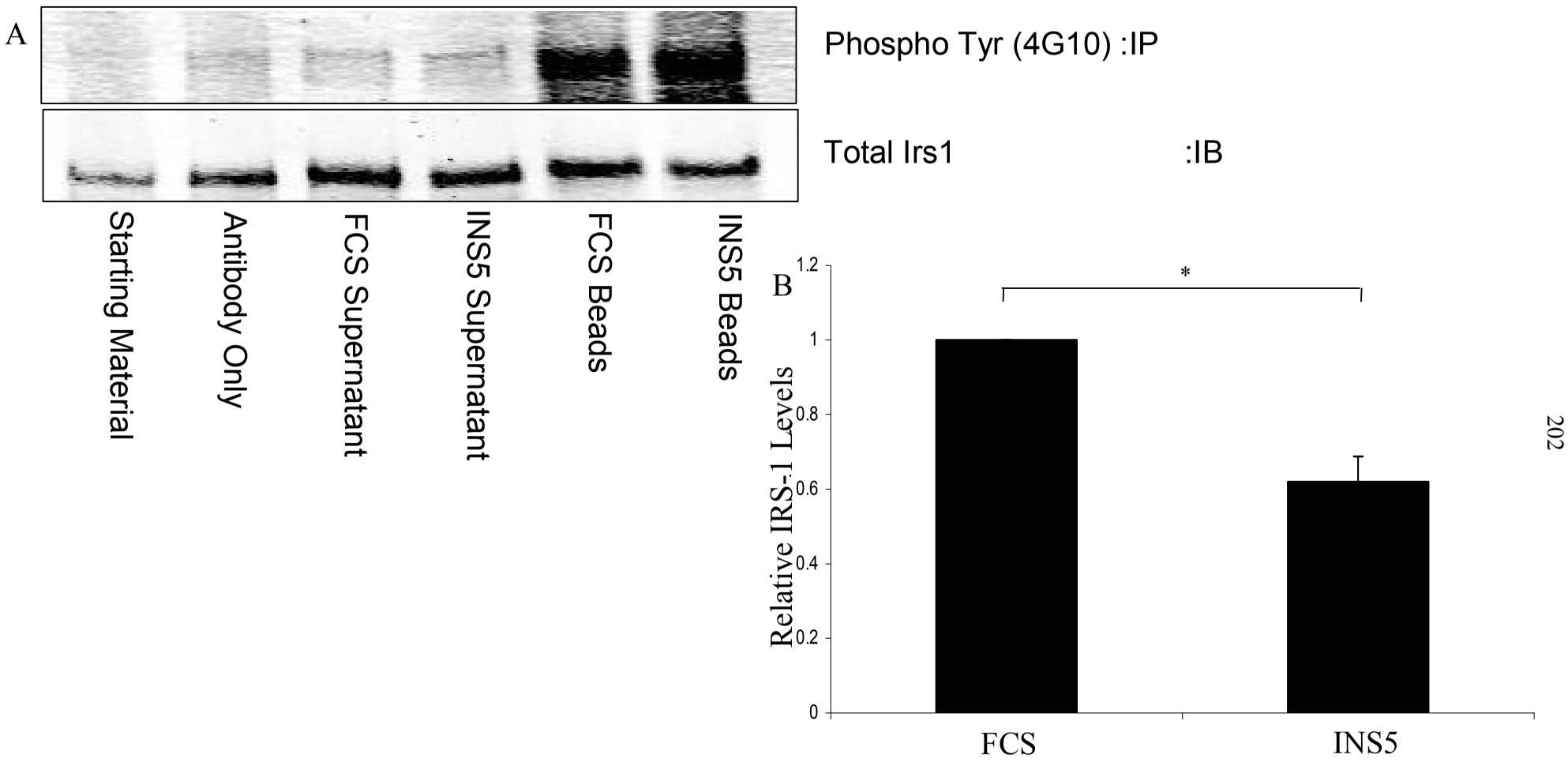


Figure 5.13 IRS-1 levels in phopshoTyr pull downs. H4IIe cells were cultured for 3 weeks in FCS + or - (INS5) 5 pM insulin. Cells were fasted for 3 hours then exposed to 1 nM insulin for 15 minutes. Tyr phosphorylated proteins were immunoprecipitated from 400 ug total protein with anti phospho Tyr (4G10) antibody before SDS PAGE then blotted with Total IRS-1 ab or 4G10. (A) Representative blot. (B) Densitometry of IP experiments. Data shown as total IRS-1 pulled down by 4G10 normalised to FCS data. Starting material = 30 µL cell lysates, antibody only = 30 µL cell lysates + 1 µg antibody, FCS / INS5 supernatant = 30 µL lysates after incubation with beads + antibody, FCS /INS5 beads = all bead bound protein n=5. * = p= 0.03.

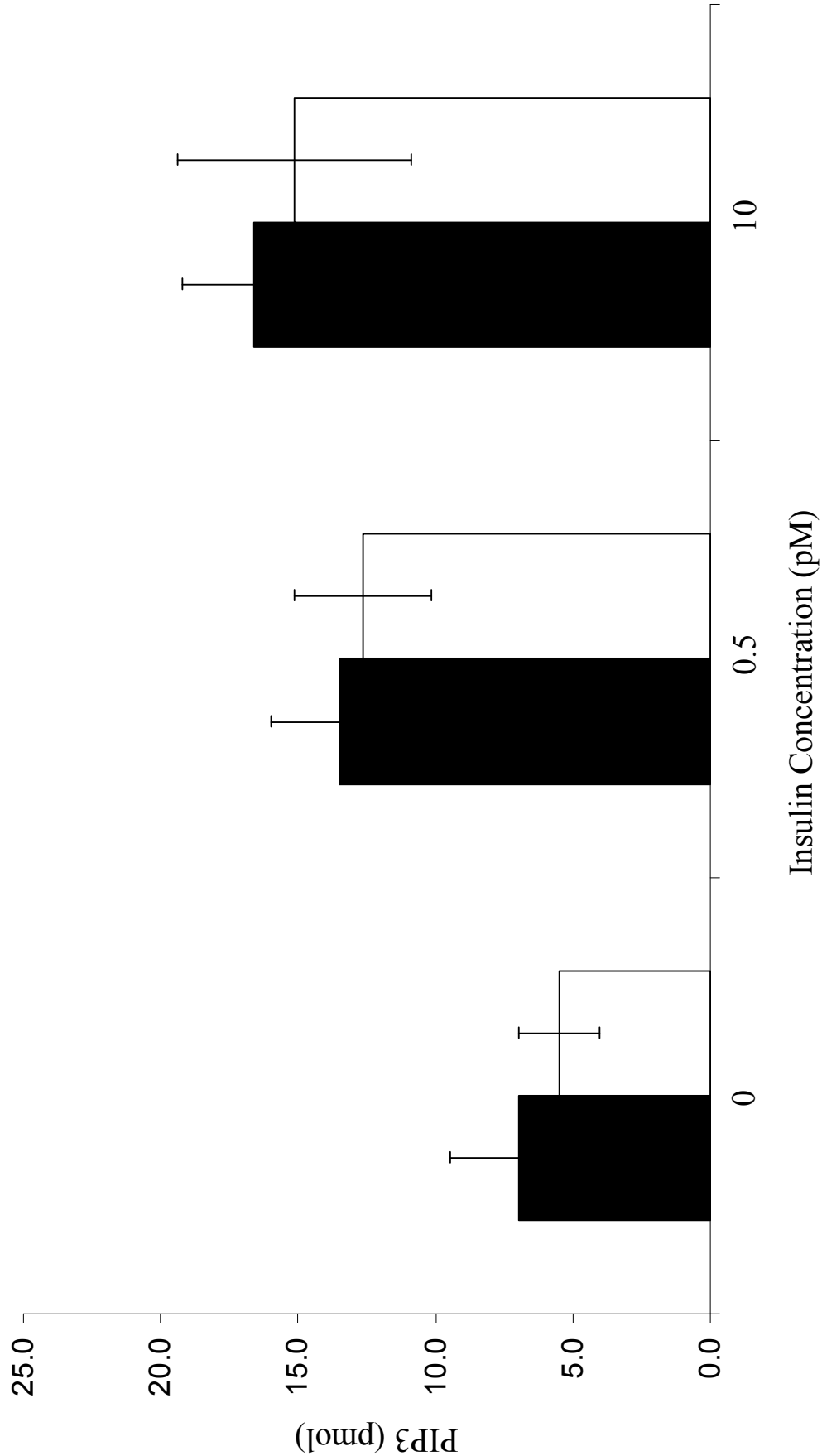


Figure 5.14 PIP3 levels in H4Ie cells cultured in FCS +/- 5 pM insulin for 3 weeks. After 3 weeks of culture cells were fasted for 16 hours prior to treatment for 10 minutes with insulin at indicated levels and cell lysis. Lysates were analysed for PIP3 levels by Dr Alexander Gray (CLS see Chapter 2.2.17). Black Bars = FCS, White bars = FCS + 5 pM insulin. (n=2)

between cells cultured in either media (Figure 5.14). Hence, if there is reduce Tyr phosphorylation of IRS-1 it is not affecting this branch of IRS-1 signalling.

5.2.5. Effects of inhibition of PKB and PI 3-kinase on PEPCK gene expression

It has been known for some time that inhibition of PI 3-kinase and PKB reduces the ability of insulin to repress PEPCK, suggesting that they are both required for this action of insulin. However it was not clear how closely associated the degree of activity of each was to the repressive action of insulin on the gene. To assess the effect that minor perturbations in the phosphorylation status of PKB may have on insulin signalling, I performed a dose response with the highly specific PKB inhibitor MK2206. H4IIe cells were fasted for 3 hours and then pre-treated for 30 minutes with increasing doses of the inhibitor. Cells were then treated with or without 10 nM insulin, cell lysates generated and probed for PKB phosphorylation (Figure 5.15 A). Even though there is a 71 to 78% reduction in the level of PKB phosphorylation with 100 to 500 nM MK2206, this has no effect on the downstream signalling to GSK-3 which maintains full phosphorylation response to insulin (Figure 5.15 A). Increasing the concentration of MK2206 to 1 μM further reduced the level of both phospho-Thr 308 and phospho-Ser473 of PKB to >82% (Figure 5.15 C). Even with this level of inhibition there was only a 16 % reduction in the phosphorylation of GSK-3 compared to control (Figure 5.15 C). A different PKB inhibitor, Akti1/2, completely abrogated the effects of insulin on phospho-Thr 308 and phospho-Ser473 PKB at 10 μM. This level of PKB inhibition reduced the phosphorylation of GSK-3 and FOXO1 to basal level (Figure 5.15 B and C). The PI 3-K inhibitor, PI-103 also reduced the phosphorylation of PKB at both Thr 308 and Ser473 sites. 1, 2 and 5 nM PI-103 reduced the phosphorylation of Thr 308 by 51.2, 53.85 and 100 % respectively. The reduction in Ser473 phosphorylation was 44.1, 51.4

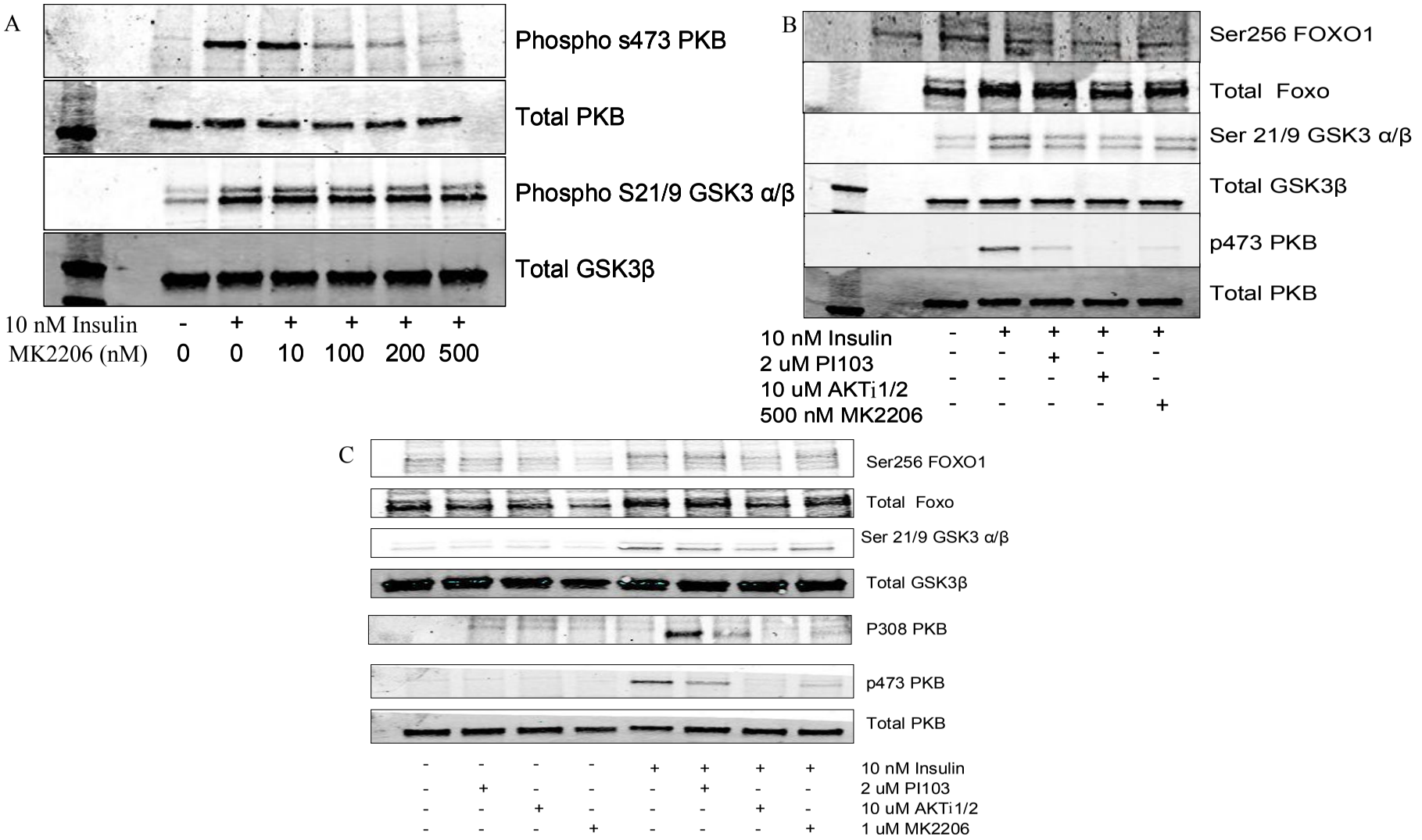


Figure 5.15 Effect of PKB inhibitors, MK2206 and AKT 1/2 and the PI3-Kinase inhibitor PI-103 on insulin signaling pathways in H4IIe cells. Cells were pre-incubated for 30 minutes in the presence or absence of indicated inhibitors, followed by a 3 hour treatment in the presence or absence of inhibitors. (A) Dose titration of MK2206 and its effects on PKB and GSK-3β phosphorylation, (B) Effect of 500 nM MK2206, 2 μM PI103 and 10 uM Akti 1/2 on PKB, FOXO1 and GSK-3β phosphorylation (C) Effect of 1 uM MK2206 , 2 μM PI103 and 10 uM Akti 1/2 on PKB, FOXO1 and GSK-3β phosphorylation. n=2 for each experiment

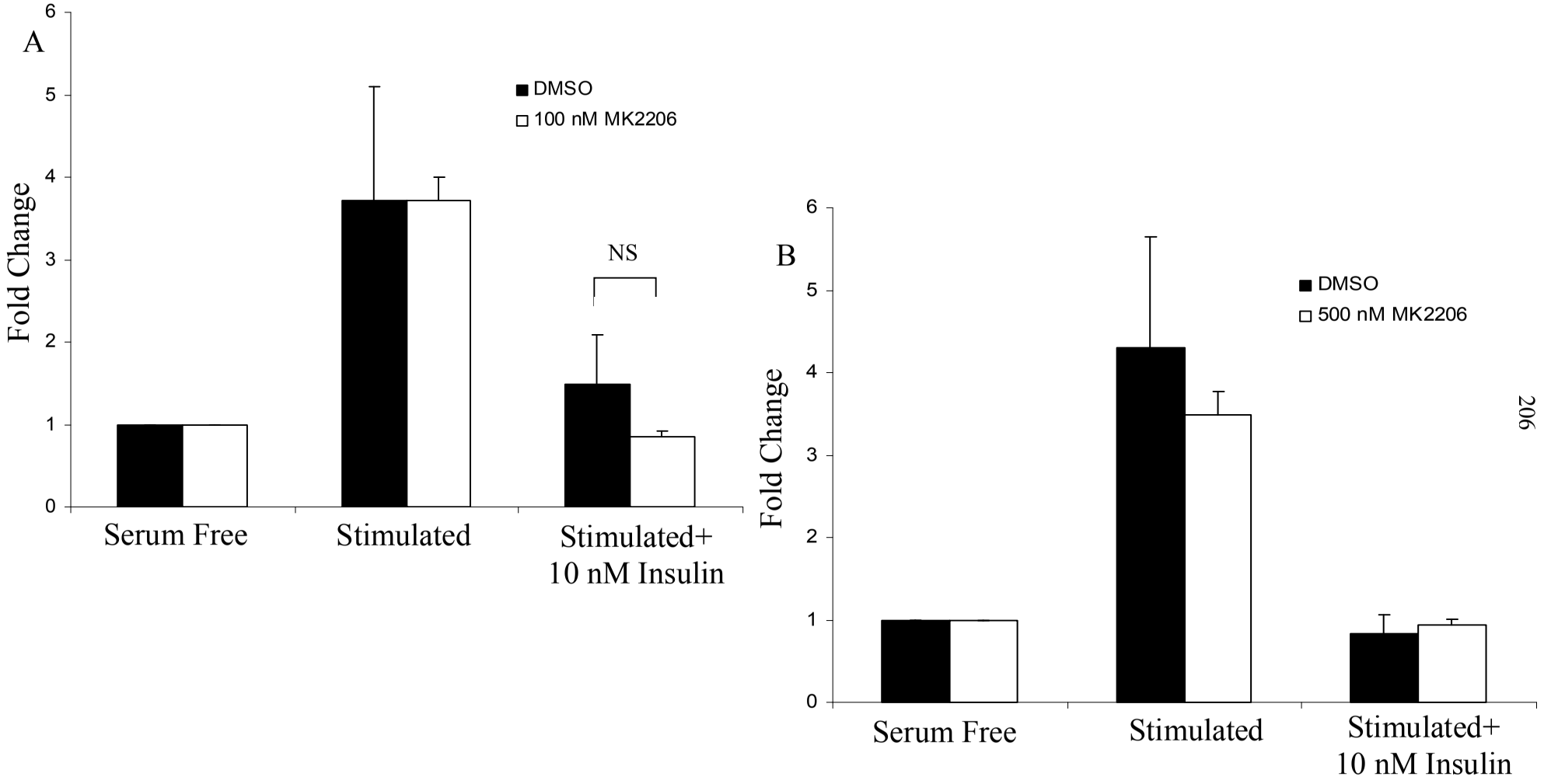
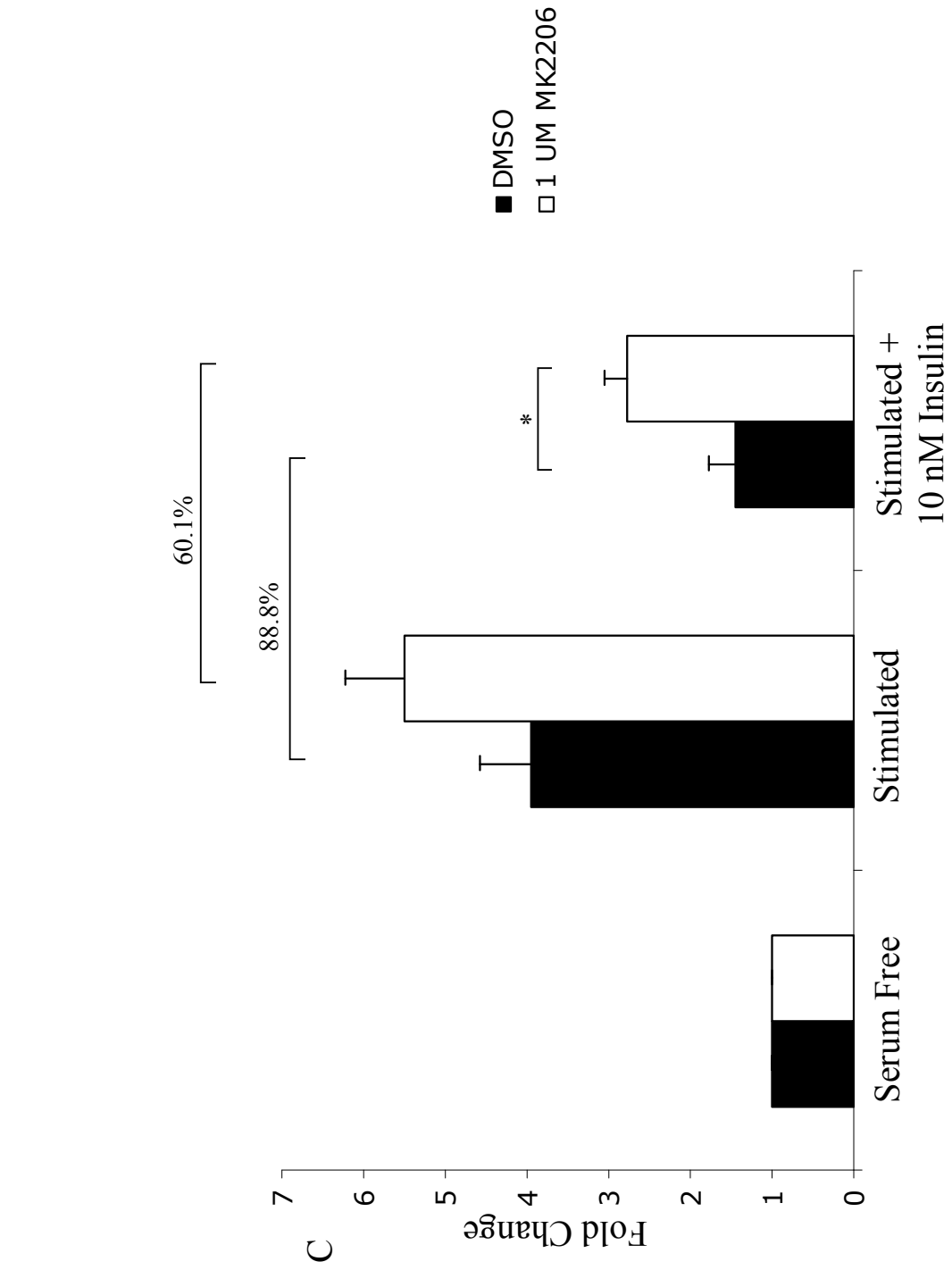


Figure 5.16 Effect of the PKB inhibitor MK2206 on PEPCK gene expression. H4IIe cells pre-incubated for 30 minutes in the presence or absence of MK2206, followed by a 3 hour treatment in the presence or absence of MK2206. Black Bars = DMSO, (A) White Bars = 100 nM uM MK2206, (B) White Bars = 500 nM MK2206, (C) White Bars = 1 uM MK2206. Data presented as fold change in ratio of PEPCK mRNA to Actin mRNA (n=2 for each experiment). P values NS=non significant *=0.02



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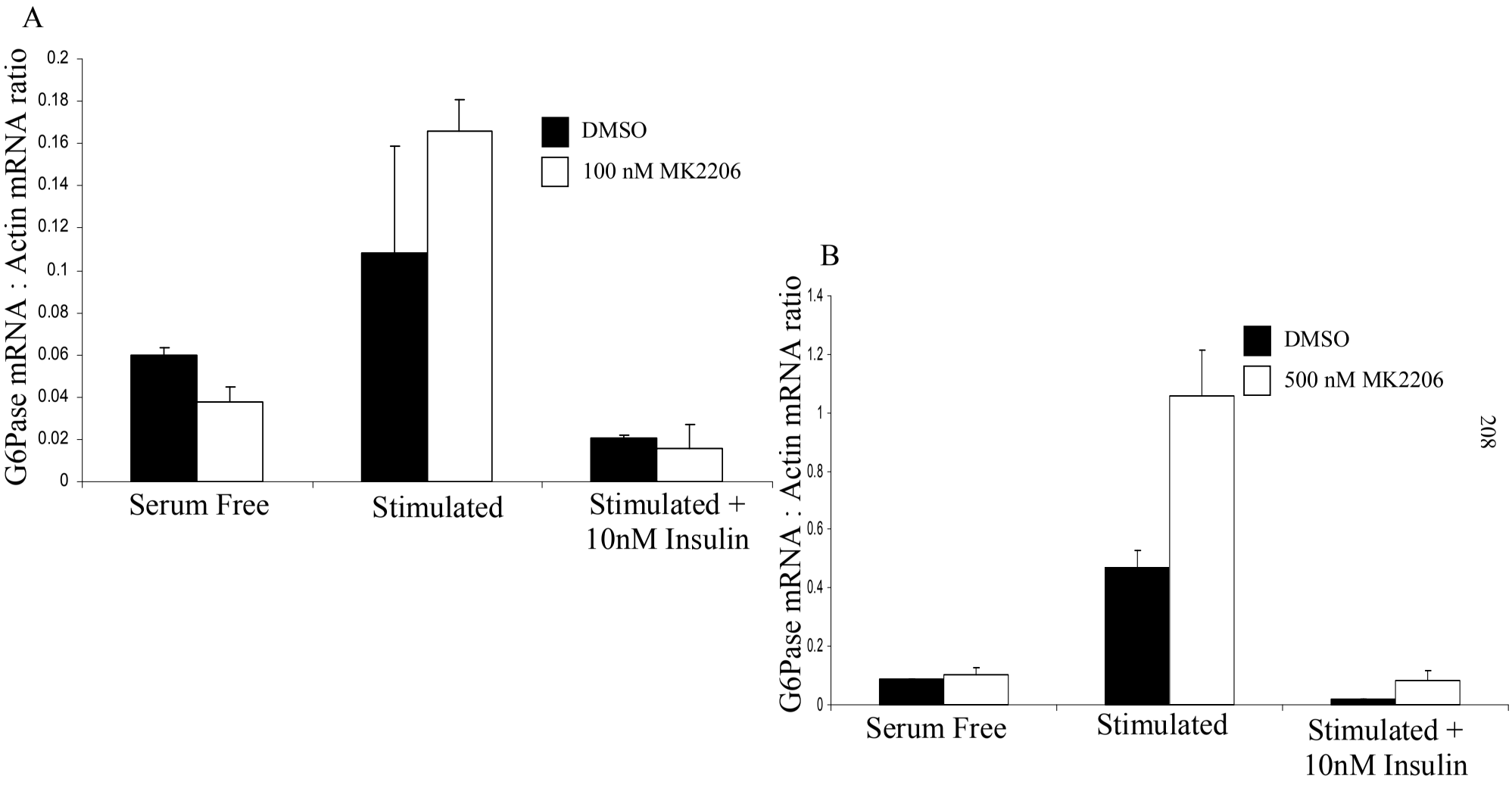
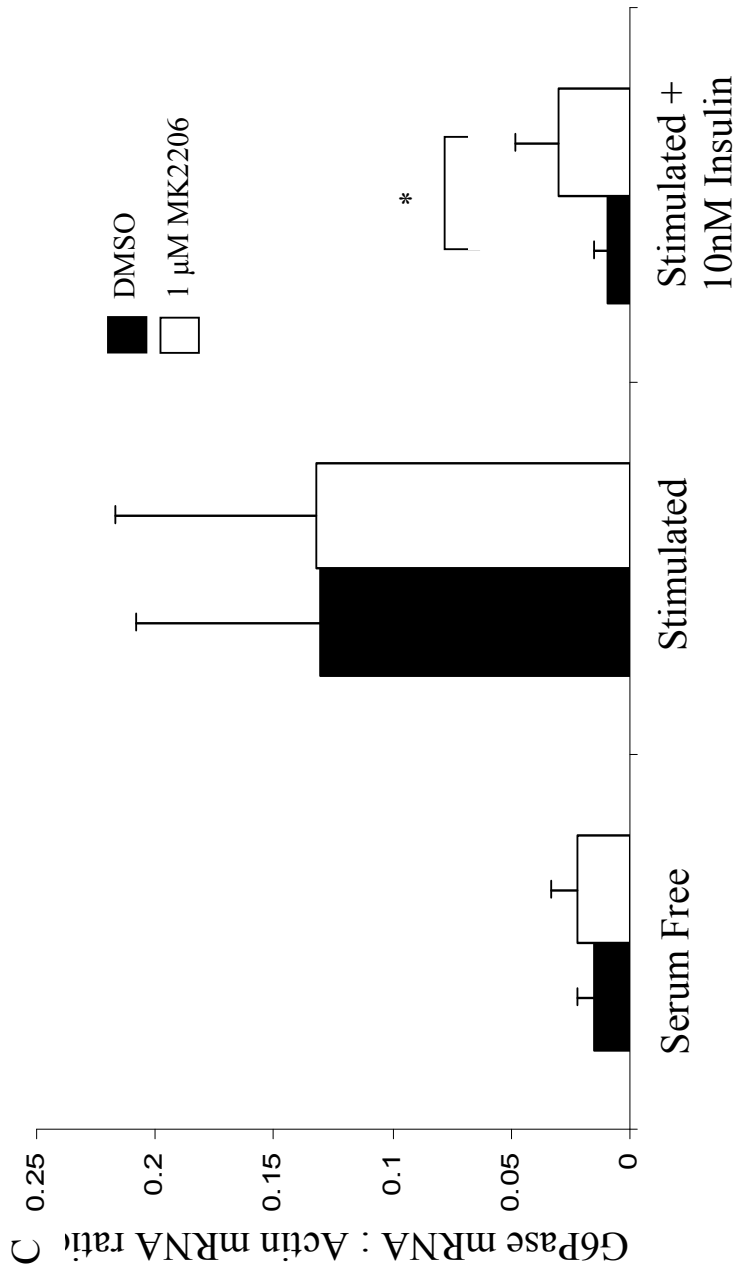


Figure 5.17 Effect of PKB inhibitor MK2206 on G6Pase gene expression. H4IIe cells were fasted for 3 hours and then pre treated for 30 minutes with or without MK2206 prior to treatment for 3 hours with dexamethasone and cyclic AMP in the presence or absence of 10 nM insulin and the presence or absence of MK2206. Data shown as ratio of G6Pase mRNA to Actin mRNA. Black bars = DMSO, (A) white bars = 100 nM MK2206, (B) white bars = 500 nM MK2206, (C) white bars = 1 uM MK2206 (n=2 for each experiment) Pvalues *=0.02



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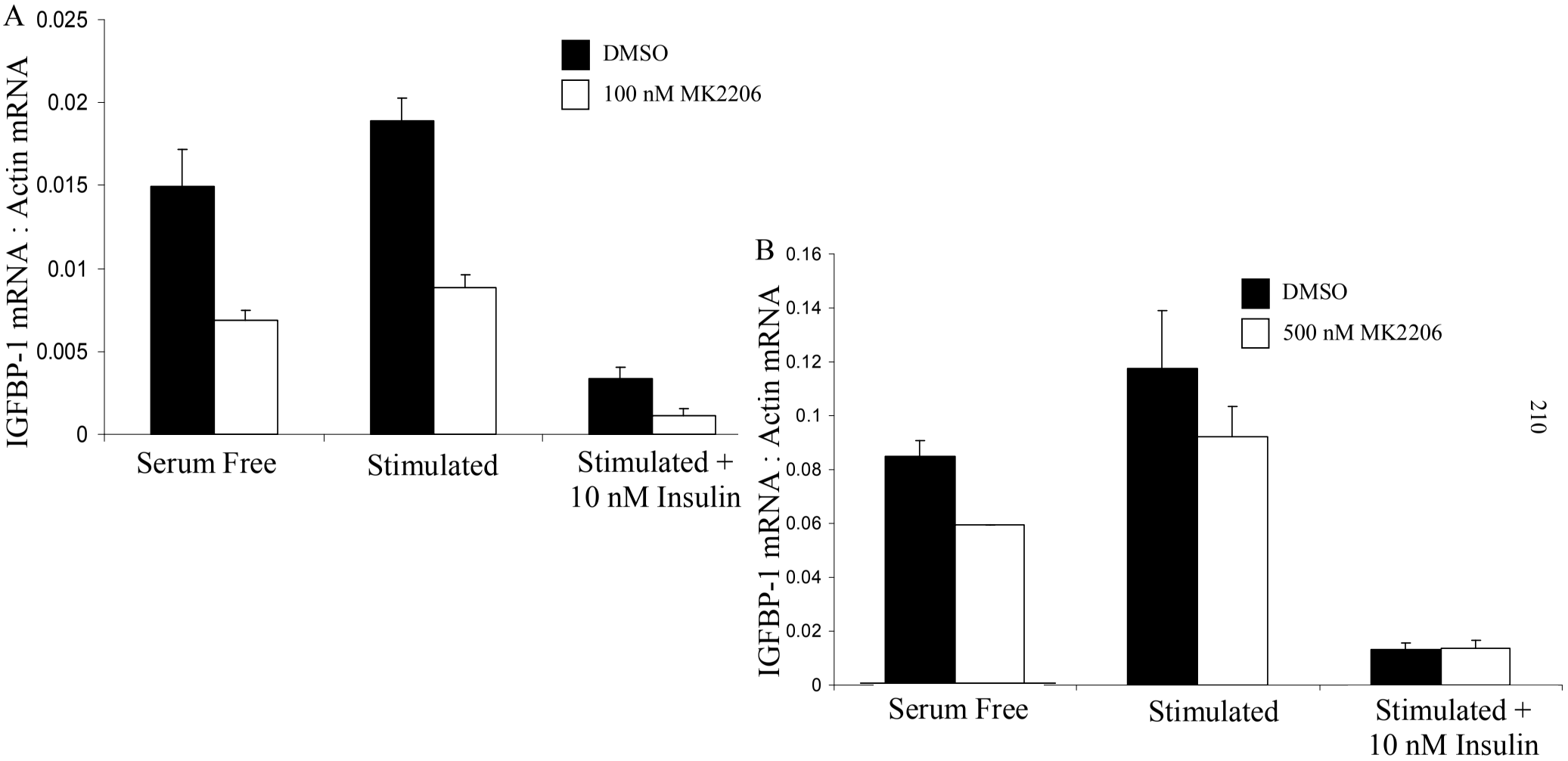


Figure 5.18 Effect of the PKB inhibitor MK2206 on IGFBP-1 gene expression. H4IIe cells were fasted for 3 hours and then pre treated for 30 minutes with or without MK2206 prior to treatment for 3 hours with dexamethasone and cyclic AMP in the presence or absence of 10 nM insulin and the presence or absence of MK2206. Data shown as ratio of IGFBP-1 mRNA to Actin mRNA. Black bars = DMSO, (A) white bars = 100 nM MK2206, (B) white bars = 500 nM MK2206, (C) white bars = 1 uM MK2206. (n=2 for each experiment)

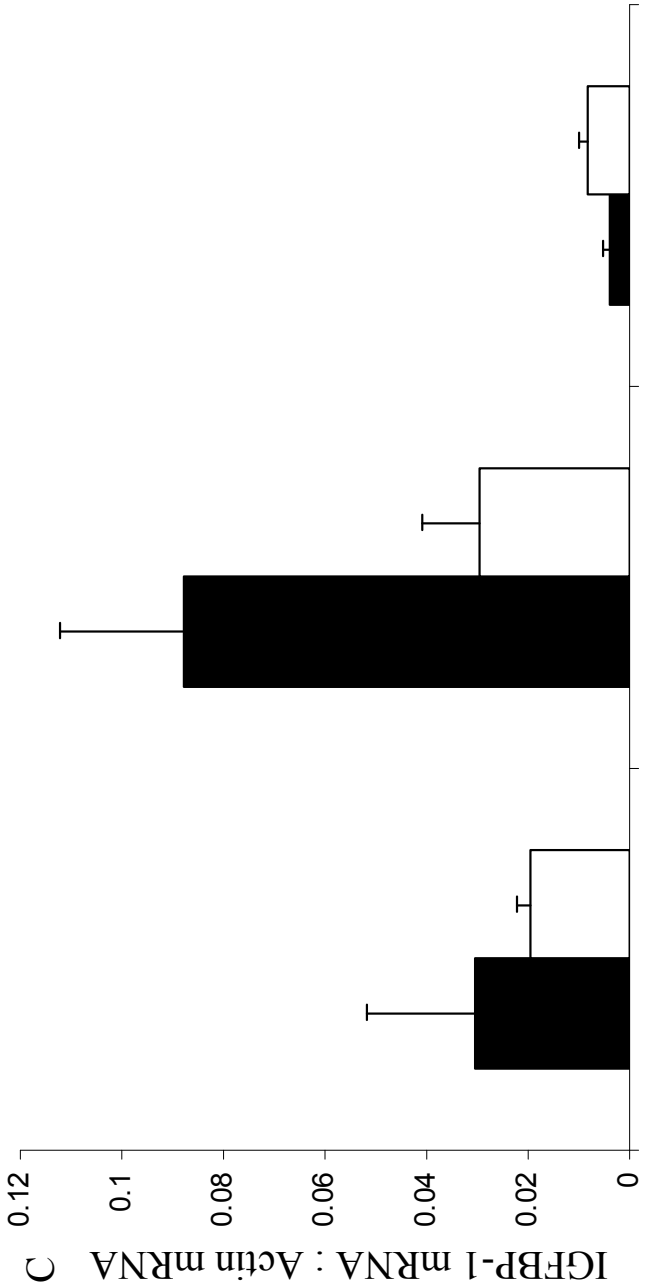


Figure 5.18 continued

and 91.7 %. Similarly insulin stimulated GSK-3 phosphorylation was reduced by 19.7, 30.0 and 81.5 % respectively (Figure 5.15 B and C).

It was of interest to examine the effect of MK2206 on the repression of PEPCK by insulin. There was no effect of MK2206 on PEPCK transcription until a concentration that promoted more than 80 % reduction in PKB phosphorylation (1 μM) was used (Fig 5.16), although this was still only partial (repression of PEPCK by 10 nM insulin from 88.8% to 60.1% ($p=0.02$)). The stimulatory effect of glucocorticoids and cAMP on PEPCK was enhanced by 1 μM MK2206 (Fig 5.16). There was no effect of 100 or 500 nM MK2206 on the ability of insulin to repress PEPCK despite partial inhibition of PKB activation.

The effects of MK2206 on G6Pase gene expression were similar to those on PEPCK. Neither 100 nor 500 nM had any effect on the insulin repression of G6Pase. However, there was a reduction in the ability of 10 nM insulin to suppress G6Pase in the presence of 1 μM MK2206 ($p=0.02$ Fig 5.17). In contrast, the addition of between 100 nM and 1 μM MK2206 reduced the stimulation of IGFBP-1 by dex/cAMP (Fig 5.18). There was no effect on the ability of 10 nM insulin to suppress IGFBP-1 gene expression (Fig 5.18).

Therefore, there is no direct correlation between the level of PKB activity and the percentage repression of PEPCK by 10 nM insulin in cells treated with different concentrations of MK2206 (Fig 5.19 A). In fact, the insulin repression of PEPCK is not affected by 60 to 80 % reduction in PKB activity, but is partially reduced when PKB phosphorylation is inhibited by more than 80% (Figure 5.19). Similarly, there is no correlation between the level of GSK-3 phosphorylation and the ability of insulin to

repress PEPCK, although perhaps surprisingly, there were very small effects of PKB inhibition on GSK-3 phosphorylation in these experiments (Figure 5.19 A).

Conversely, PI 3-K inhibition by PI-103 (from 1 to 5 μM) produced a more graded inhibition of insulin induced phosphorylation of PKB at both Thr 308 and Ser473 (Fig 5.20), and of phosphorylation of GSK-3 α and β at Ser9/21 (Fig 5.20). This allowed a direct comparison of the degree of pathway inhibition with the regulation of PEPCK expression by insulin (Fig 5.21). For example, the addition of 1 μM PI-103 reduces the percentage repression of PEPCK by 10 nM insulin from 98.7% to 58.5%, while 2 μM PI-103 reduces it to 56.5% and 5 μM PI-103 reduces this still further to 40.9% (Fig 5.21 B). The reduction in the level of phosphorylation of PKB at Ser473 at these concentrations of PI-103 is 44.1, 51.4 and 91.7% respectively, and this correlates with the repression of PEPCK by insulin (Fig 5.22 A). Indeed, the line of best fit has an r^2 value of 0.94. The level of phospho-Thr 308 of PKB correlates just as well as Ser473 with the level of PEPCK suppression by insulin, and produces an r^2 value of 0.94 (Figure 5.22 B). Together the data strongly suggests a relationship between the level of PEPCK suppression and the inhibition of signalling downstream of PI 3-kinase. That said, the level of GSK-3 phosphorylation downstream of PI 3-kinase inhibition did not correlate very closely with PEPCK repression by insulin (Figure 5.22 C), although 5μM PI-103 had a much greater effect on GSK-3 phosphorylation than MK2206, and at that concentration repression of PEPCK was blunted (Figure 5.22 C).

It was surprising that selective inhibition of PKB using MK2206 had only a partial effect on insulin regulation of PEPCK transcription (Figure 5.16). Previous reports had suggested that the Ras-MAP kinase pathway can substitute for the PKB pathway and repress PEPCK gene transcription under certain circumstances. Therefore I investigated

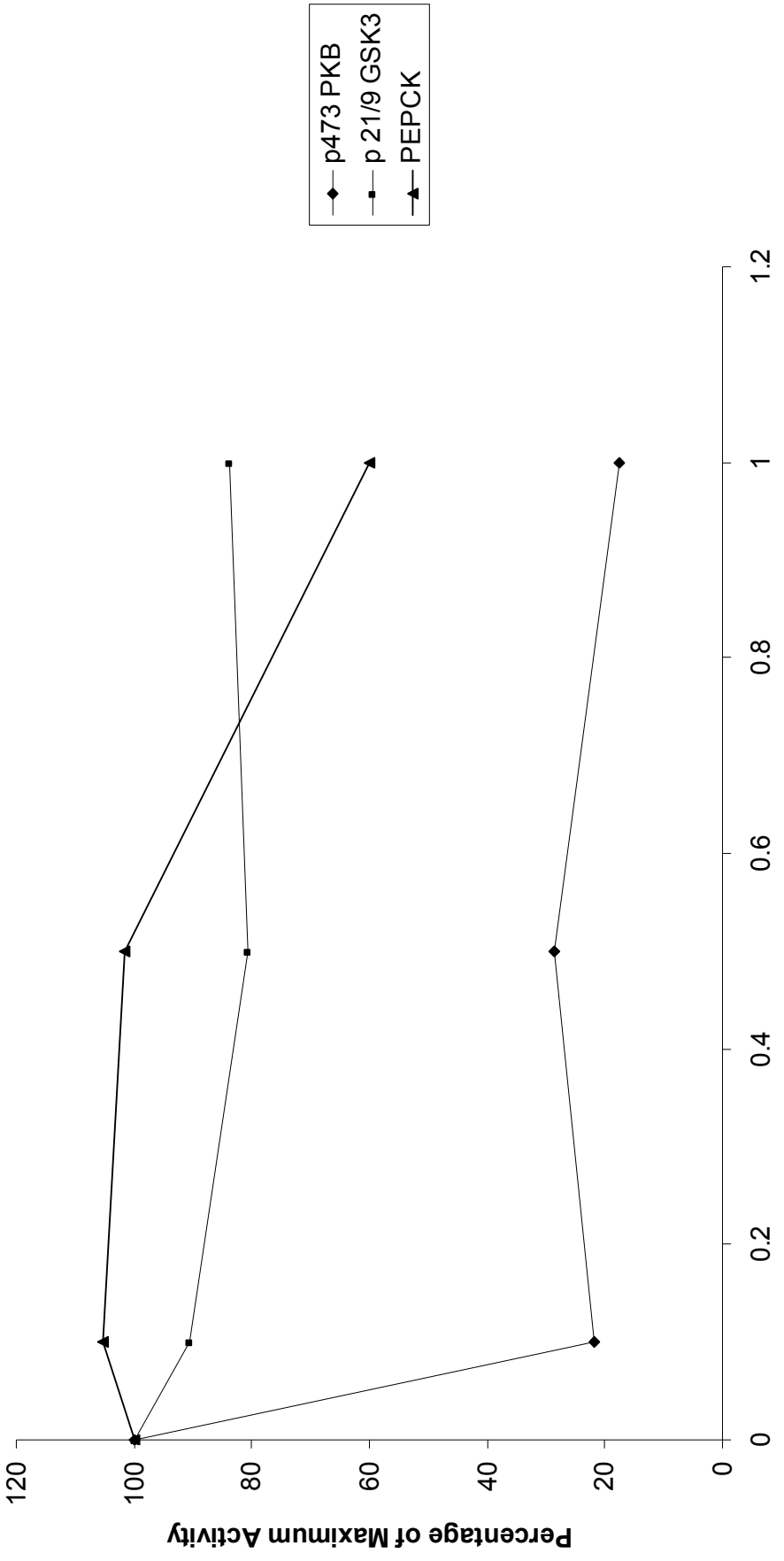


Figure 5.19 There is no correlation between the inhibitory effects of MK2206 on PKB and GSK-3 phosphorylation and the repression of PEPCK by 10 nM insulin. Percentage of maximum activity of insulin on p473 PKB, p21/9 GSK-3 and PEPCK (n=2).

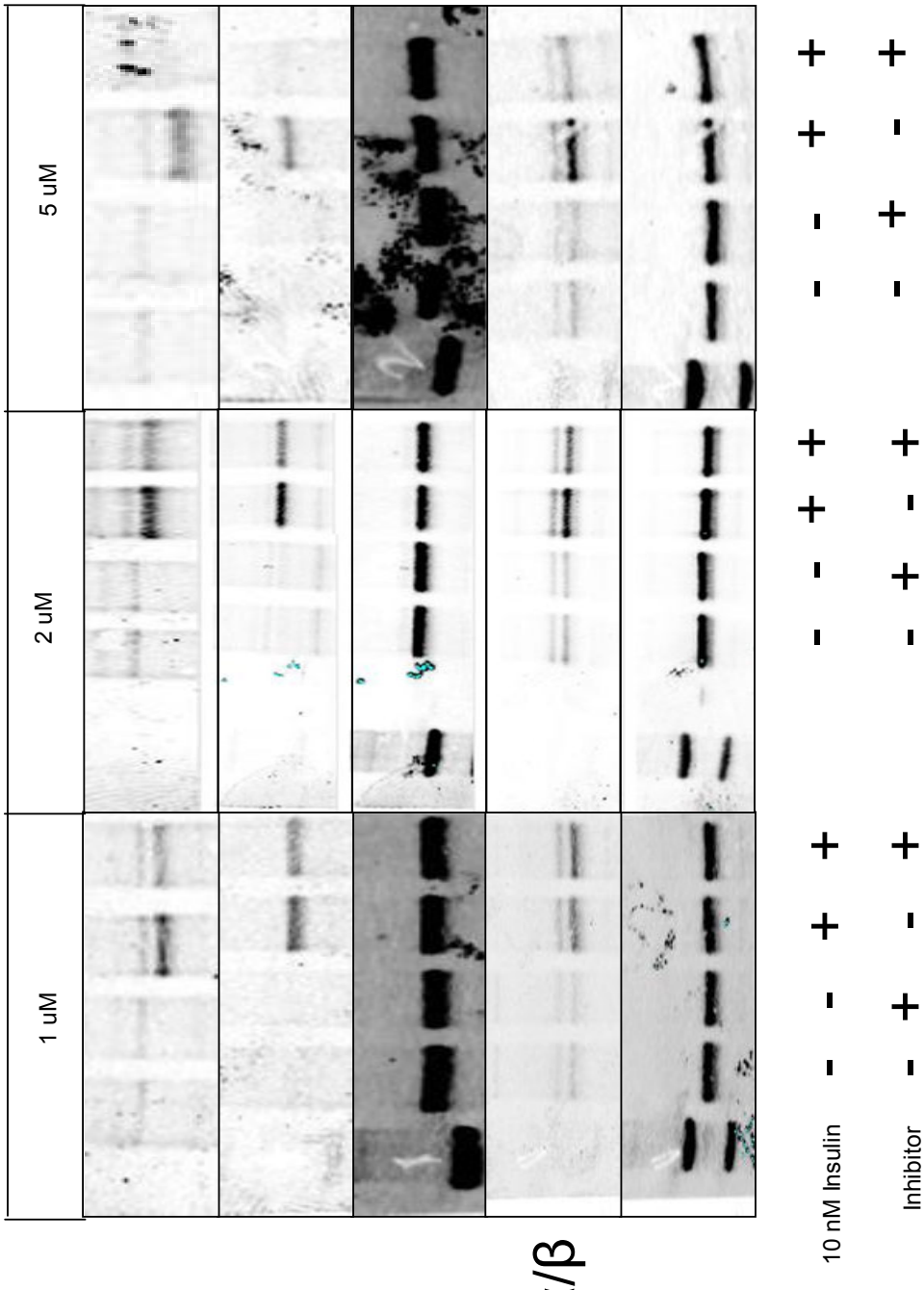
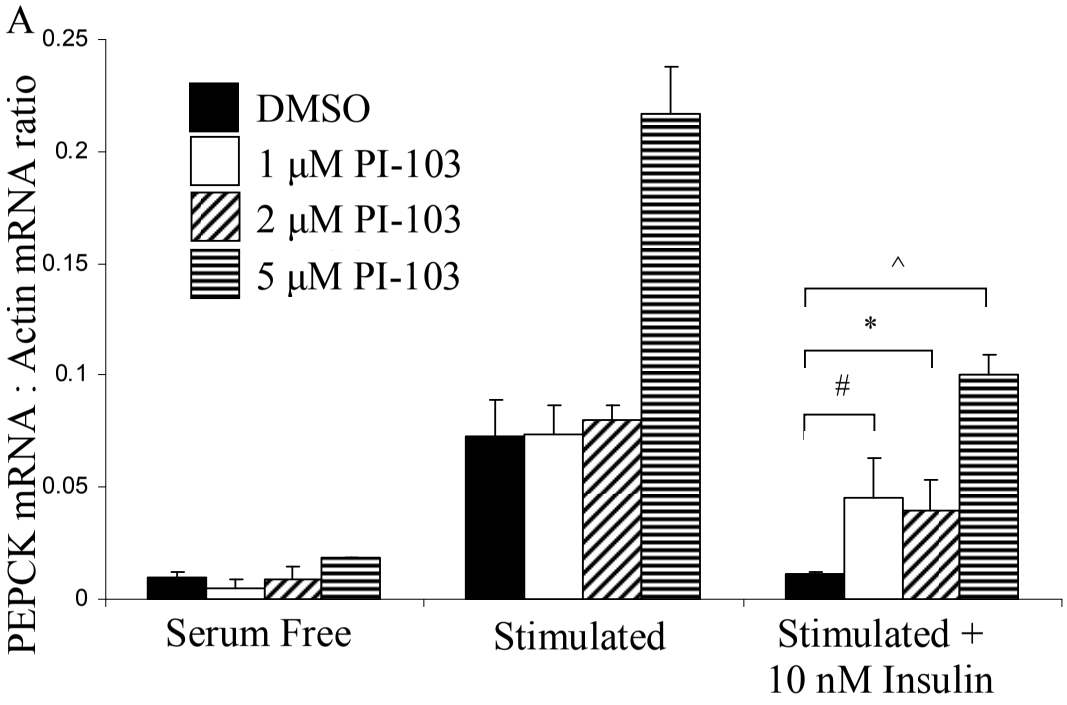


Figure 5.20 Effect of PI103 on the insulin signalling pathway in H4IIe cells. Cells were pre-incubated for 30 minutes in the presence or absence of PI103, followed by a 1 hour treatment in the presence or absence of insulin ± PI103. Protein lysates were generated and PKB and GSK-3β phosphorylation assessed by Western blot using the antibodies indicated (n=2).



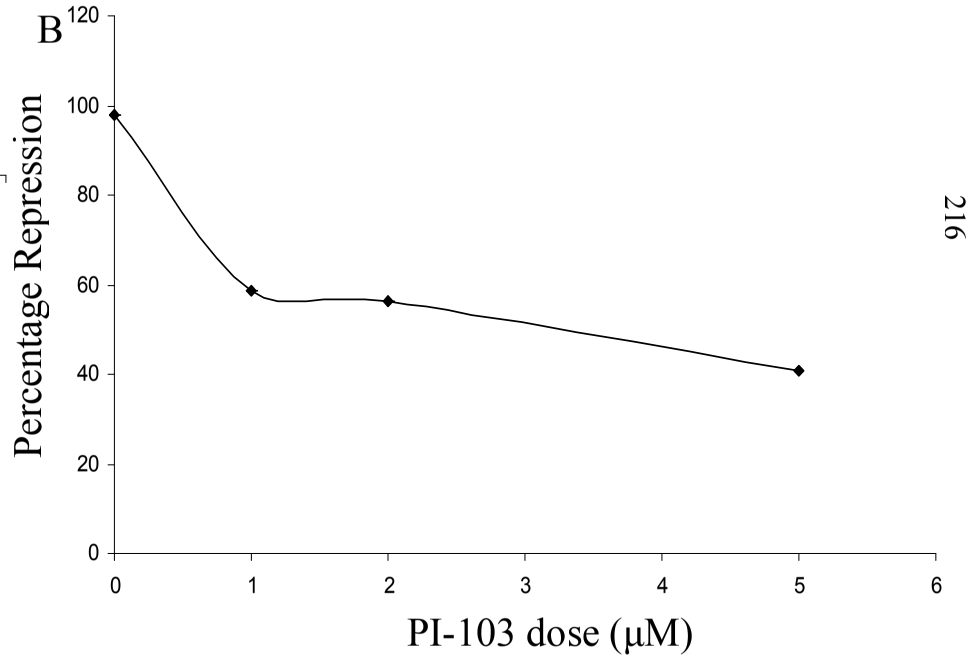
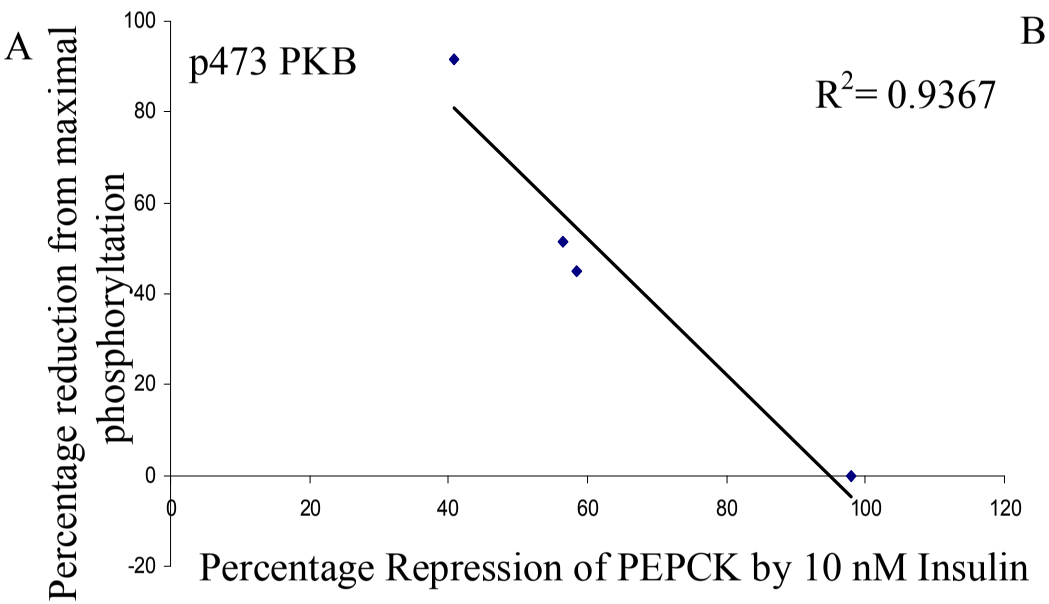
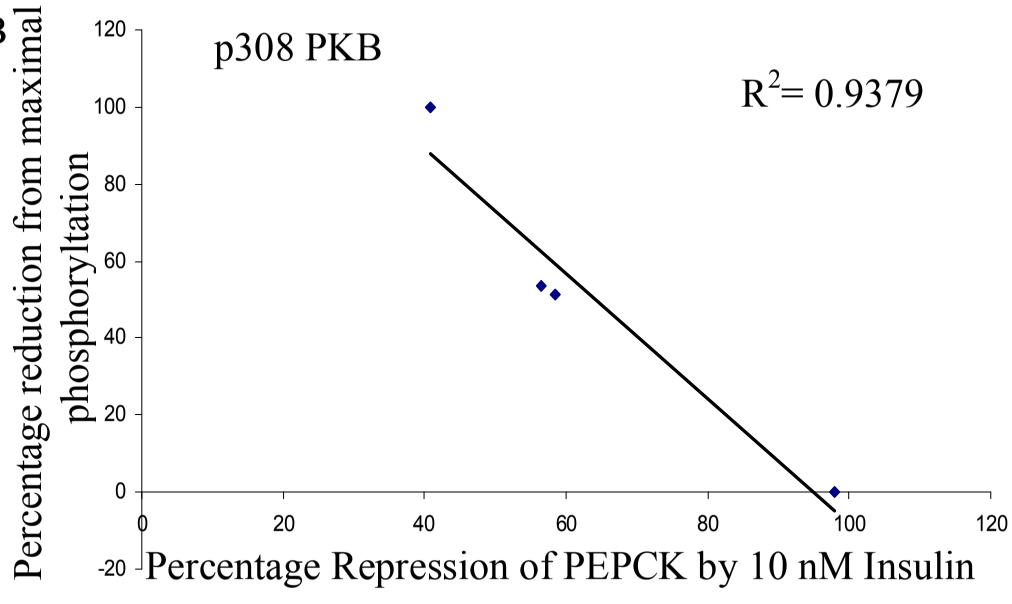


Figure 5.21 Effect of PI3 Kinase inhibition with PI-103 on PEPCK gene expression in H4IIe cells. Cells were fasted for 3 hours prior to 30 minutes pre-treatment in the presence or absence of PI-103. Cells were then treated for 3 hours as indicated and analysed by Taqman (A). Data shown as PEPCK mRNA : Actin mRNA ratio. Black bars = DMSO, White bars = 1 uM PI-103, Diagonal Lines = 2 uM PI-103, Horizontal bars = 5 uM PI-103. (B) Percentage repression of PEPCK by 10 nM insulin compared to dose of PI-103 (n=2).p values ^=<0.001, #=0.03,*=0.04.





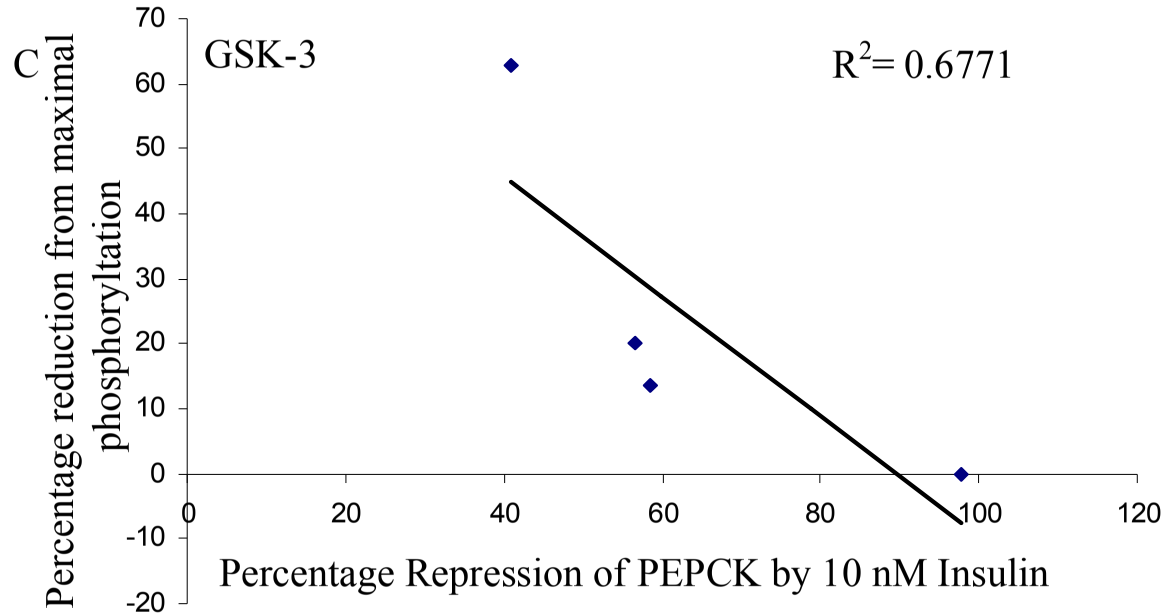
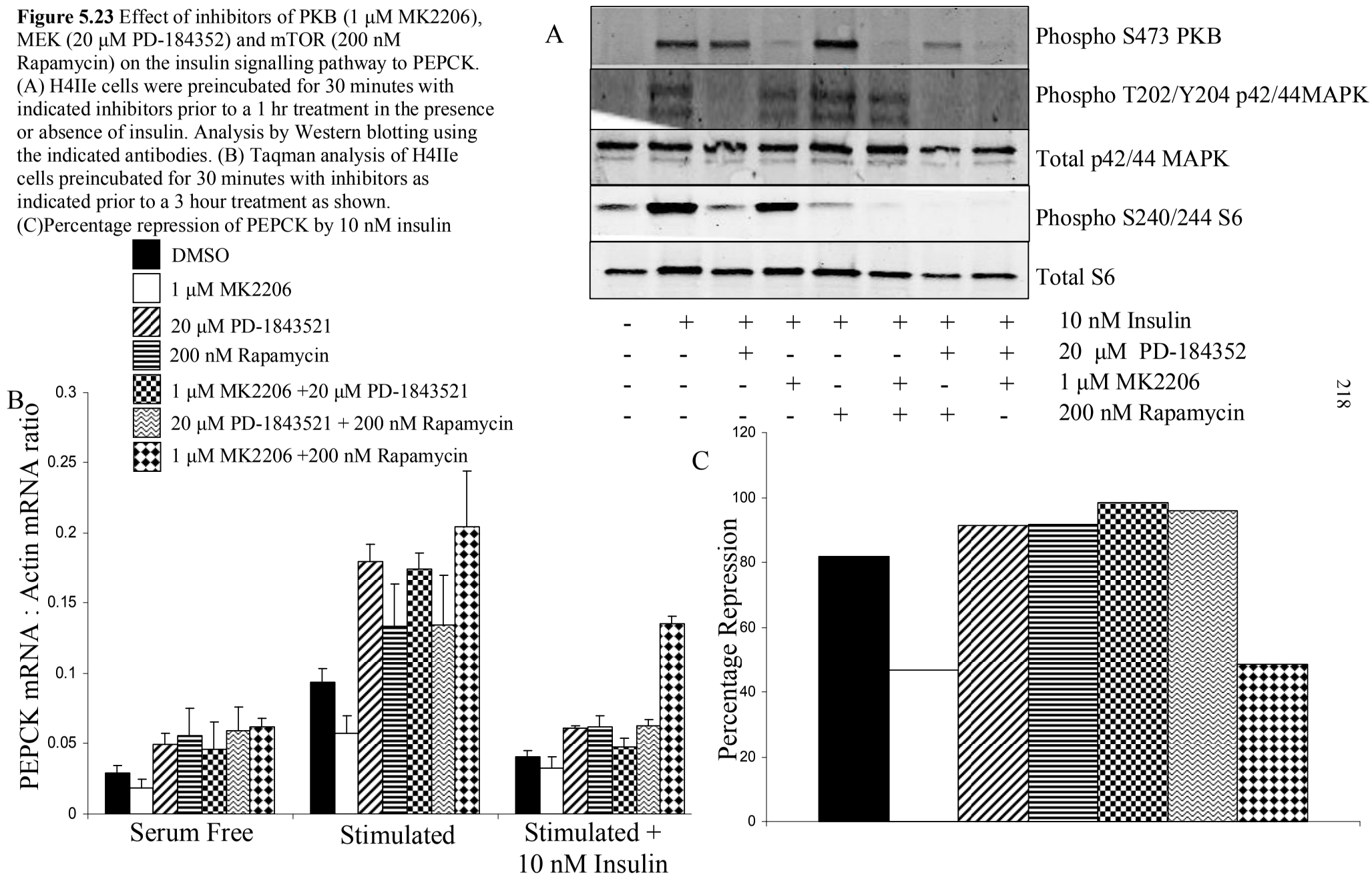


Figure 5.22 Correlation of activation of PKB by insulin as measured by PKB phosphorylation at S473 (A) and PKB phosphorylation at T307 (B) with repression of PEPCK by 10 nM insulin. (C) Correlation of GSK-3β phosphorylation at Ser9 and repression of PEPCK by 10 nM insulin. (D) Insulin repression of PEPCK is reduced by the presence of PI103.

Figure 5.23 Effect of inhibitors of PKB (1 μM MK2206), MEK (20 μM PD-184352) and mTOR (200 nM Rapamycin) on the insulin signalling pathway to PEPCK. (A) H4IIe cells were preincubated for 30 minutes with indicated inhibitors prior to a 1 hr treatment in the presence or absence of insulin. Analysis by Western blotting using the indicated antibodies. (B) Taqman analysis of H4IIe cells preincubated for 30 minutes with inhibitors as indicated prior to a 3 hour treatment as shown. (C)Percentage repression of PEPCK by 10 nM insulin



whether these different pathways were redundant by exposing cells to inhibitors of each of these pathways (PKB, MEK and mTOR) simultaneously and checked whether increasing the concentration of MK2206 even further could completely blunt insulin regulation of the gene. H4IIe cells were incubated with MK2206, PD-184352 and rapamycin in the presence of insulin (Fig 5.23). In each case I confirmed efficacy of inhibitor treatment, for example MK2206 reduced Ser473 of PKB phosphorylation but not phosphorylation of S6 ribosomal protein or p42/44 MAP kinase in response to insulin (Fig 5.23 A). There was, however, a reduction in phosphorylation of S6 in the presence of either rapamycin or PD-184352 (Fig 5.23 A). This may reflect the fact that phosphorylation of Ser440/444 by S6K is dependent on prior phosphorylation at Ser235/236 by RSK, downstream of p42/44 MAPK. However the effect of PD-184352 on S6 phosphorylation was enhanced by MK2206 (Fig 5.23 A). The insulin-induced phosphorylation of p42/44 MAPK was reduced by PD-184352 (Fig 5.23 A). It was not possible to treat cells with all 3 inhibitors without substantial cell loss.

Once more MK2206 (1 μM) reduced basal and stimulated levels of PEPCK but also reduced insulin regulation of this gene (Fig 5.23 B). The presence of PD-184352 or rapamycin increased the stimulation of PEPCK mRNA level even in the presence of PKB inhibition (Fig 5.23 B). Dual inhibition of mTOR and PKB, with rapamycin and MK2206, did not have any greater effect on insulin regulation of PEPCK than MK2206 alone (Fig 5.23 B and C). Most interestingly, dual inhibition of PKB and p42/44 MAPK by MK2206 with PD-184352 appeared to reverse the effect of MK2206 alone on this gene (Fig 5.23 B and C). It is difficult to understand why loss of MEK activation of p42/44 MAP kinase would overcome the need for PKB in insulin regulation of PEPCK gene transcription.

5.3. Discussion

The work in this chapter attempted to create a more simplified cell based model of insulin resistance which would further knowledge in 3 main areas;

- To identify whether insulin was the factor in human diabesity serum that promoted insulin resistance in chapter 3,
- To identify the insulin signalling changes that occurred during the generation of insulin resistance, and
- To create a version of the cell model for compound screening that did not require collection and storage of human diabesity serum.

5.3.1. Is insulin the factor responsible for the effect of diabesity serum on H4IIe cells?

There are clearly many differences in the components of the diabesity and control sera (Table 3.1). Many signalling pathways have inherent feedback controls that prevent chronic activation, for example adrenergic receptors are down regulated following stimulation. Therefore one could envisage that prolonged exposure to insulin could generate downregulation of insulin signalling and hence insulin resistance. The mean insulin concentration in control sera was 39 pM, while in diabesity sera it was 105 pM, around 2.7 times higher. Therefore the cells cultured in diabesity sera were exposed to 2.7 times higher insulin than those in control sera. Of course, as I dilute the sera to a final concentration of 5% (v/v) in the culture media this means the cells were only exposed to around 5 pM insulin, even with the diabesity sera. This is still much lower than normal fed levels of plasma insulin (up to 5000 pM in the portal circulation), although cells in the body will not be chronically exposed to this level of insulin. Using culture media supplemented with 5 pM insulin led to relatively poor insulin sensitivity

of the H4IIe cells compared to cells grown in control levels of insulin, at least as measured by insulin repression of PEPCK gene transcription. The magnitude of insulin resistance generated by addition of 5 pM insulin to FCS was greater than that seen in cells cultured in diabesity serum and also than that in cells cultured in supplemented human serum. There is no significant difference in the levels of PEPCK in either basal or stimulated conditions in the studies with supplemented calf sera. There are a couple of differences in the PEPCK responses in the studies with human serum. Firstly, culturing the cells in 5 pM insulin increased the level of PEPCK expression under all conditions. Despite this there was still a relative loss of insulin sensitivity in cells exposed to the chronic insulin. Secondly, the actual concentration at which the insulin does response shifted was different in the human sera studies (0.5 to 1 nM as opposed to 0.1 to 0.5 nM in the calf sera cells). This may reflect the fact that the basal insulin levels between the calf and human sera are different or other factors present in the sera are having effects on insulin sensitivity, which is something that could easily be investigated.

Interestingly, there is a differential effect of this cell culture manipulation on G6Pase and PEPCK response to insulin. This is despite the fact that the EC50 for the effects of insulin on both genes lies between 0.1 and 0.2 nM. It is known that both genes require PI 3-K activity for full suppression (Dickens et al., 1998, Miyake et al., 2002, Sutherland et al., 1995). However, the same cannot be said for PKB. There are conflicting results for the effects of PKB on PEPCK. It has been shown that dominant negative PKB mutants do not reduce the ability of insulin to suppress PEPCK, but also that expression of a tamoxifen regulated active PKB mimics the action of insulin by preventing gene induction by glucocorticoids and cAMP (Kotani et al., 1999, Liao et al., 1998). The effects on G6Pase are somewhat different, with overexpression of PKB only

partially affecting G6Pase transcription (Schmoll et al., 2000). This suggests that PI3-Kinase is a critical signalling node in the insulin suppression of both genes, but that other downstream targets of PI 3-K have a role to play in the suppression of G6Pase. In addition only a small activation of PKB is required to mediate its effects on PEPCK, explaining the need for almost full inhibition in my MK2206 studies.

It has been known for some time that hyperinsulinaemia can lead to the development of insulin resistance (Rizza et al., 1985). Insulin is usually secreted in a pulsatile nature, and this is tightly controlled in ~4 minute bursts (Song et al., 2000). The insulin levels seen in the portal circulation range from ~200-500pM in the fasting state and from 1000 to 5000 pM in the fed state (Song et al., 2000, Porksen et al., 1996). Insulin sensitivity naturally decreases with age, and this is overcome by an increase in β-cell mass (Matveyenko et al., 2008). Conversely, in type 2 diabetes, there is approximately a 65% reduction in β-cell mass (Butler et al., 2003) and these β-cells are also deficient in insulin secretion losing their pulsatile nature, but overall releasing higher levels of insulin particularly in the fasting state. An increase in the intraportal level of insulin can also induce insulin resistance (Marban and Roth, 1996, McGuinness et al., 1990) even if there is an increase in the level by only 50% (McGuinness et al., 1990).

This raises the distinct possibility that a β-cell defect in insulin secretion, causing even mild hyperinsulinemia, may actually be the first step in the development of insulin resistance. The offspring of patients with type 2 diabetes show both insulin resistance and β-cell dysfunction with higher insulin levels (Stadler et al., 2009). However, this β-cell dysfunction is still present in those who are insulin sensitive (Stadler et al., 2009). Similarly, patients with insulinomas, which gives a persistent basal level of hyperinsulinaemia, are also insulin resistant (Nankervis et al., 1985). All of this data

together does indicate that loss of proper control of insulin secretion may be an initiator of many of the defects found in type 2 diabetes and if true calls into question the rationale of treating the disease with insulin secretagogues. In fact it is possible that what is actually required are interventions that reduce insulin secretion at the early stage of the disease, or alternatively restore normal control of secretion. There is clearly also an issue with proper control of insulin therapy in Type 1 diabetes to reduce the risk of insulin resistance associated with the use of long acting insulins.

Furthermore, if mild continuous hyperinsulinaemia can lead to insulin resistance then this should be taken into account when developing dietary advice for the prevention of diabetes. For example if one is continually snacking (or consuming high sugar drinks) and therefore chronically delivering glucose to the pancreas, this will lead to a more persistent level of insulin delivery to the liver and the subsequent development of insulin resistance. Indeed, gluconeogenesis is suppressed less by insulin in those with physiological hyperinsulinaemia compared to controls. This higher glucose output is maintained in both the fasting and fed state leading to higher glucose output in all states (Gastaldelli et al., 2001). The snacking behaviour of populations has increased over recent years, and this may go some way to explaining the increase in type 2 diabetes in industrialised countries.

5.3.2. What molecular changes occur in cells cultured in low insulin for 3 weeks?

The insulin resistance measured using the PEPCK gene promoter could in theory be due to a defect anywhere along the signalling pathway from receptor to gene promoter. The insulin receptor influences insulin sensitivity in a number of ways. Alterations in receptor number, affinity or signal transmission can all change insulin sensitivity of a given cell. The affinity of the receptor for insulin decreases as insulin levels increase

due to negative co-operativity (DeMeyts et al., 1976), and there is also a switch from the high affinity receptor type to a lower affinity receptor type after exposure to higher insulin levels (Seino and Bell, 1989). This aspect was not investigated in my studies but would be well worth checking, although downstream readouts (e.g. PKB) appeared relatively normal in the cells with insulin resistance. As with other hormone receptors, continued exposure to hormones leads to a down-regulation of extracellular receptors, both *in vitro* and *in vivo* (Caro et al., 1987, Gavin et al., 1974). In the presence of hyperinsulinaemia the Tyr kinase activity of the activated insulin receptor can be reduced due to negative feedback from Ser phosphorylation (Zick et al., 1983), the increased action of phosphatases on the receptor itself (Kusari et al., 1994) and the effects of negative regulators of insulin signalling such as SOCS1 (Krebs and Hilton, 2003).

Likewise, feedback phosphorylation of IRS-1 at Ser307 in response to prolonged insulin signalling reduces its ability to associate with the insulin receptor (Aguirre et al., 2002). There are many proposed IRS-1 Ser kinases, including JNK (Aguirre et al., 2000, Lee et al., 2003), m-TOR (Carlson et al., 2004), S6K (Harrington et al., 2004), GSK-3 (Eldar-Finkelman and Krebs, 1997) and ERK (De Fea and Roth, 1997) all proposed to couple prolonged signaling to downregulation of signaling and potentially, if not controlled, leading to insulin resistance. I have shown that not all insulin signalling kinases are equally insulin sensitive with S6 being approximately 20 and 42 times more sensitive to insulin than ERK or PKB. Therefore, chronic low concentrations of insulin may affect the signalling network, without direct effects on each kinase. However, I found no evidence of down regulation of either insulin receptors or IRS-1, as total basal and insulin stimulated PIP-3 levels were equal between insulin sensitive and insulin resistant cells and overall there was no difference in IRS-1 Tyr phosphorylation or Ser307

phosphorylation. However, it is worth noting that the cells had to be fasted for 16 hours prior to PIP3 analysis, during which time any deficits created by exposure to supplemented medium may have diminished.

Therefore, I was unable to find any significant alterations in the sensitivity of the cells to insulin using these signaling components as readouts. It is possible that the chronic exposure to insulin has altered something more fundamental in the regulation of the PEPCK gene promoter (e.g. level of a transcription factor or an epigenetic regulation). Alternatively it could be simply that the sensitivity of PEPCK mRNA to changes in cellular insulin action is much greater than that of the signaling pathways or that the Taqman method is more sensitive at detecting small dynamic changes than semi-quantitative Western blotting. In order to establish whether small changes in signaling would actually translate to changes in the regulation of the PEPCK gene promoter I then tried to establish the degree of change in the activity of signaling nodes that would be required to alter insulin sensitivity of the gene promoter.

5.3.3. How great a change in signaling is required to affect regulation of PEPCK by insulin?

I found that there is very little effect of preventing as much as 80% of potential PKB activation on the repression of PEPCK gene transcription by insulin. Concentrations of the PKB inhibitor MK2206 that achieved ~90% reduction in potential PKB phosphorylation did reduce insulin repression of the PEPCK gene. This is consistent with previous work where expression of a dominant negative PKB had no effect on the expression of PEPCK (Kotani et al., 1999) and where a different PKB inhibitor (Akti 1/2) had no effect on insulin regulation of PEPCK until there was complete suppression of PKB phosphorylation (Logie et al., 2007). Therefore, this suggests that major loss of

PKB activation by insulin would be required for this to be the molecular deficit responsible for the insulin resistance produced in my cell model, and such changes in PKB phosphorylation should certainly be evident.

On the other hand, by using a PI 3-kinase inhibitor I was able to show a good correlation between the level of pathway activation and the level of PEPCK suppression by insulin. This suggests that the activity of PI 3-kinase is paramount in determining the translation of the strength of the signal from the insulin receptor to the gene. In agreement with this, a dominant negative PI 3-kinase mutant blocks insulin inhibition of PEPCK, and a constitutively active mutant behaves like an insulin mimetic, reducing PEPCK gene transcription (Kotani et al., 1999). All of this together suggests the change in insulin sensitivity of the PEPCK gene promoter after incubation in diabesity sera or 5 pM insulin for 3 weeks could be due to reduced PI 3-kinase signaling. However, I could not detect changes in the insulin sensitivity of any of the major downstream signalling pathways in these cells. Interestingly incubation of cells with a PKB inhibitor in combination with either an mTOR or ERK inhibitor showed no greater effect on insulin regulation of PEPCK gene transcription than with a PKB inhibitor alone. Therefore, it is quite possible that although the PI 3-kinase pathway is required for this action of insulin, there is an as yet unidentified pathway or mechanism independent of PI 3-kinase signaling that is responsible for the development of insulin resistance.

5.3.4. Potential mechanisms of insulin resistance:

Mitochondrial dysfunction is proposed to be involved in the development of type 2 diabetes, while in high fat feeding models of type 2 diabetes oxidative stress is evident in the liver (Raffaella et al., 2008). However, hyperglycaemia itself is considered to be the major driver of the development of oxidative stress. That said, since insulin

resistance is evident years before the hyperglycaemia that defines overt diabetes it is unlikely that if oxidative stress is a cause of insulin resistance that high glucose is the driving factor in its creation. Early in the development of reduced insulin sensitivity hyperinsulinaemia develops to overcome it. Furthermore, in animal experiments of hyperinsulinaemia in the absence of hyperglycaemia, there is increased oxidative stress in the absence of an increased inflammatory response (Ling et al., 2007). Clinically, hyperinsulinaemia is often a necessary part of the treatment of diabetes, and it has been known from the early days of insulin therapy that decreasing insulin dosages can actually improve hyperglycaemia, thereby by definition improving insulin sensitivity (Somogyi and Kirstein, 1938). In adipose tissue, chronic insulin treatment induces the development of reactive oxygen species (ROS) and these impair insulin signalling and subsequently glucose uptake (Ge et al., 2008). However, there was no impairment of insulin signalling in my H4IIe cells. It is possible to measure oxidative stress by assaying the levels of carbonyl groups introduced into proteins by ELISA or Western blotting. Other possible methods to assess oxidative stress in response to 5 pM insulin include measuring superoxide dismutase activity or Tyr nitrosylation.

If hyperinsulinaemia is a cause of insulin resistance, then the current treatment of type 2 diabetes with insulin, particularly in a non pulsatile nature, may contribute further to insulin resistance and be inadvertently worsening complications, albeit less than those caused by uncontrolled hyperglycaemia. Indeed, insulin therapy with long acting insulin analogues have been implicated in the development of certain cancers (Hemkens et al., 2009, Jonasson et al., 2009, Colhoun, 2009) and relief of insulin resistance with metformin can reduce cancer risk in type 2 diabetes (Evans et al., 2005). The problems of hyperinsulinaemia could be confounded if all tissues are not equally resistant. For example, if the brain remains insulin sensitive in the presence of peripheral resistance,

then insulin therapy may lead to an increase in Alzheimer's dementia (Carro and Torres-Aleman, 2004).

However, reactive oxygen species are not necessarily detrimental to health. Indeed, reactive oxygen species are generated in adipocytes after exposure for insulin and the hydrogen peroxide generated is essential for downstream intracellular insulin signalling (Mahadev et al., 2001b, Mahadev et al., 2001a). In *C. elegans*, caloric restriction, which is concomitant with a decrease in insulin signalling, leads to a prolonged lifespan despite the greater oxidative stress due to higher levels of oxidative phosphorylation, and the prolongation of lifespan can be reduced by the addition of antioxidants (Schulz et al., 2007). It is important to note that the higher levels of ROS in this model are not in the presence of hyperglycaemia or hyperinsulinaemia. Nevertheless, the *C. elegans* analogue of AMPK, AAK-2, is required for lifespan prolongation (Schulz et al., 2007). In human insulin resistance when hepatocytes have more than sufficient energy, one could assume that glycolysis could supply the ATP required for cell metabolism. In the presence of metformin, which depletes cellular ATP (Foretz et al., 2010), more ATP will be required for cellular metabolism, i.e. the cell is in a state of relative energy restriction. This will cause a shift towards oxidative phosphorylation to make up the ATP debt, and therefore the production of ROS and an improvement in morbidity and mortality.

An alternative explanation for the rather PEPCK focused effects of 5 pM insulin exposure would be changes in the levels, activity or localization of one or more transcription factors involved in the control of PEPCK gene transcription. PGC1α directly binds to HNF4α and promotes PEPCK expression. Overexpression of PGC1α increases hepatic glucose production through upregulation of PEPCK gene transcription

(Puigserver et al., 2003), and the effect of PGC1α on PEPCK is prevented by phosphorylation of FOXO (Hall et al., 2000). Insulin induction of SREBP-1 also represses PEPCK transcription (Chakravarty et al., 2001). Indeed there are a host of transcription factors known to bind to the PEPCK gene promoter including FOXA2, CREB, C/EBPβ, RXR, CAR, HNF1, TORC, CBP and of course the basal RNA polymerase II complex. It would be well worth examining whether chronic hyperinsulinaemia affects the expression or binding to the PEPCK gene promoter of these transcription factors in any way. Unfortunately it is not yet entirely clear which transcription factors are key to insulin repression of the gene promoter although FOXO, FOXA2, PGC1α, TORC and CBP are the most likely to influence insulin regulation.

5.4. Conclusions

I have induced insulin resistance in H4IIe cells by culturing in the presence of 5 pM insulin implying that chronic raised insulin is potentially the serum factor in diabesity that promotes hepatic resistance to itself. A similar approach should be used to examine the effects of simply adding the other factors that are raised in human diabesity serum to culture medium to assess if they lead to the development of insulin resistance. The development of insulin resistance can be treated in two ways, either overwhelming the resistance with more insulin, which may be detrimental, or by relieving the insulin resistance. The latter approach would seem more logical. Currently weight loss or use of the biguanides can improve insulin sensitivity. The mechanism by which these drugs promote insulin sensitivity is still unknown, and investigation into one possible mechanism forms the basis of the following chapter.

Chapter 6. Molecular connections between the insulin sensitising actions of metformin and DNA repair.

6.1. Introduction

The beneficial effects of biguanides on the symptoms of diabetes have been known for over 500 years. The isolation of guanidine from the French lilac led to the discovery of phenformin, no longer used due to the high risk of lactic acidosis, and subsequently metformin. Metformin remains the first line pharmacological agent in the treatment of type 2 diabetes and the most commonly prescribed oral anti-hyperglycaemic agent. The use of metformin continues to increase despite the fact that the mechanism of action remains uncertain and somewhat controversial. In an attempt to find mediators of metformin efficacy Pearson and colleagues recently performed a genome wide association study and surprisingly implicated the DNA repair enzyme, ATM (mutated in ataxia telangiectasia), in the glucose regulating actions of metformin (Zhou et al., 2011). The only previous indication that ATM may influence metformin action was the molecular connections between ATM and the metformin induced kinase, AMPK (discussed in Chapter 1.11).

ATM is a Ser/Thr protein kinase activated in response to double stranded DNA breaks thus allowing either DNA repair or cell cycle arrest and apoptosis. The substrates of ATM are numerous but include SMC1, CHK1 and -2, BRCA1, NBS1, p53 and FANC-D2, all of which are involved in genomic integrity (Kitagawa et al., 2004, Kim et al., 1999a, Osborn et al., 2002). Therefore individuals with mutations in ATM, a condition known as ataxia telangiectasia (A-T), are more sensitive to ionising radiation leaving them susceptible to the development of carcinomata (Savitsky et al., 1995). Most interestingly, the families of patients with A-T have been reported to have a higher incidence of insulin resistance (Bar et al., 1978, Blevins and Gebhart, 1996). The ATM/p53 signalling pathway has long been associated with DNA repair and hence in the suppression of tumours but more recently has been linked to regulation of glucose

homeostasis (Armata et al., 2010) and there is increasing awareness of the links between metabolism and cell growth (Najafov and Alessi, 2010).

In addition to ATM, the related DNA repair kinase molecule, DNA-dependent protein kinase (DNA-PK), has recently been implicated in the control of glucose metabolism through the regulation of gluconeogenic gene expression. Upon feeding, DNA-PK is dephosphorylated and activated (Wong et al., 2009). DNA-PK is also activated by AMPK, a major downstream target of biguanides (see Chapter 1.11 and 1.12.2.1 and (Chanda et al., 2009)). DNA-PK then phosphorylates upstream stimulatory factor-1 (USF-1) which induces the transcription of SHP (Chapter 1.10.6) leading to the repression of G6Pase and PEPCK gene transcription along with an increase in lipogenesis (Wong et al., 2009, Kong et al., 2011, Chanda et al., 2009). Therefore, it seems that cell cycle proteins are responsive to nutritional cues and hence contribute to the balanced regulation of gluconeogenesis and energy homeostasis.

The energy balance within cells is maintained by cellular respiration. This is the method by which energy is liberated as ATP to fuel intracellular processes and occurs due to 2 catabolic processes, glycolysis and oxidative phosphorylation. Glycolysis splits the glucose into two 3 carbon sugars liberating 2 molecules of ATP in the process. The products of glycolysis can then be used in oxidative phosphorylation yielding up to a further 34 molecules of ATP. The rate of oxidative phosphorylation is decreased under hypoxic condition by the induction of hypoxia-inducible factors (HIFs), and these transcription factors can also increase gluconeogenic gene expression (Choi et al., 2005). In LKB-1 and AMPK deficient fibroblasts, the levels of HIF-1α are also increased (Shackelford et al., 2009) suggesting a role for this pathway in the maintenance of

oxidative phosphorylation and also for a role of biguanides in the regulation of cellular respiration.

The work in this chapter is an attempt to obtain mechanistic information on the link between ATM and metformin control of glucose production in the liver. In particular I investigate the possible role of DNA repair proteins in the control of AMPK and gluconeogenic gene expression by biguanides.

6.2. Results

6.2.1. ATM and gluconeogenic gene expression

To assess the influence of ATM on a well reported action of metformin and AMPK, namely the control of hepatic gene expression, this was monitored in H4IIe cells exposed to Ku55993, a specific inhibitor of ATM. Metformin stimulated the phosphorylation of AMPK and ACC within 30 minutes (Figure 6.1), while pretreatment of the cells with Ku55993 reduced both basal and metformin stimulated phosphorylation of AMPK and ACC (Figure 6.1). The inhibitor reduced the basal level of SMC phosphorylation (a reported substrate of ATM), and although metformin did not stimulate the phosphorylation of SMC, this phosphorylation appeared less sensitive to KU55993 in the presence of metformin (Figure 6.1). Ku55993 also reduced basal and insulin stimulated phosphorylation of S6 (Figure 6.1). Etoposide, a compound often used to induce double stranded DNA breaks and hence activate ATM, had no effect on SMC, AMPK or ACC phosphorylation in my experiments (Figure 6.1).

These experiments verified that metformin could induce AMPK under these conditions and that the relatively selective ATM inhibitor could reduce a number of signalling events involved in insulin action. Next H4IIe cells were fasted for 3 hours prior to 3 or 16 hours stimulation with dexamethasone (500 nM) and cAMP (100 μM 8-CPTcAMP) in the presence or absence of insulin, metformin or etoposide (although I had no evidence that etoposide could activate ATM in these cells). Total cellular RNA was extracted and cDNA synthesised before assessment of PEPCK and actin levels by Taqman analysis. At both time points, there was stimulation of PEPCK expression by dexamethasone and cAMP and this stimulation was prevented by the presence of insulin (Figure 6.2). There was no effect on hormonal regulation of PEPCK expression by culturing in the presence of metformin or etoposide (Figure 6.2).

Metformin is a much weaker inducer of AMPK in cells than phenformin (Jalling and Olsen, 1984). Therefore it was possible that the regulation of PEPCK by AMPK required a more robust induction of this pathway than could be achieved by overnight exposure of cells to metformin. Hence, the optimal concentration of phenformin required to induce phosphorylation of AMPK was established (Figure 6.3). Phenformin (0.25mM) induced AMPK 3-fold, compared to a 1.3-fold rise with 2.5 mM metformin (Figures 6.1 and 6.3). Interestingly, there was a similar rise in the phosphorylation of ACC with both agents (Figure 6.3).

H4IIe cells were fasted for 3 hours prior to 3 hours stimulation with dexamethasone (500 nM) and cAMP (100 μM 8-CPTcAMP) in the presence or absence of insulin or phenformin. Total cellular RNA was extracted and cDNA synthesised before assessment of PEPCK and actin levels by Taqman analysis. PEPCK expression was stimulated by dexamethasone and cAMP and this was prevented by culturing in the presence of either insulin or phenformin (Figure 6.4). Therefore phenformin had a greater effect on AMPK phosphorylation and prevented stimulation of PEPCK (Figures 6.3 and 6.4). Thus, the effects of inhibition of ATM were assessed on these actions of phenformin. There was a trend towards phenformin stimulation of AMPK phosphorylation being reduced by the presence of Ku55993, but this was not statistically significant (Figure 6.5), mainly due to the fact

that the data was quite variable between experiments even on the degree of induction in AMPK phosphorylation by phenformin. Again, the insulin stimulated phosphorylation

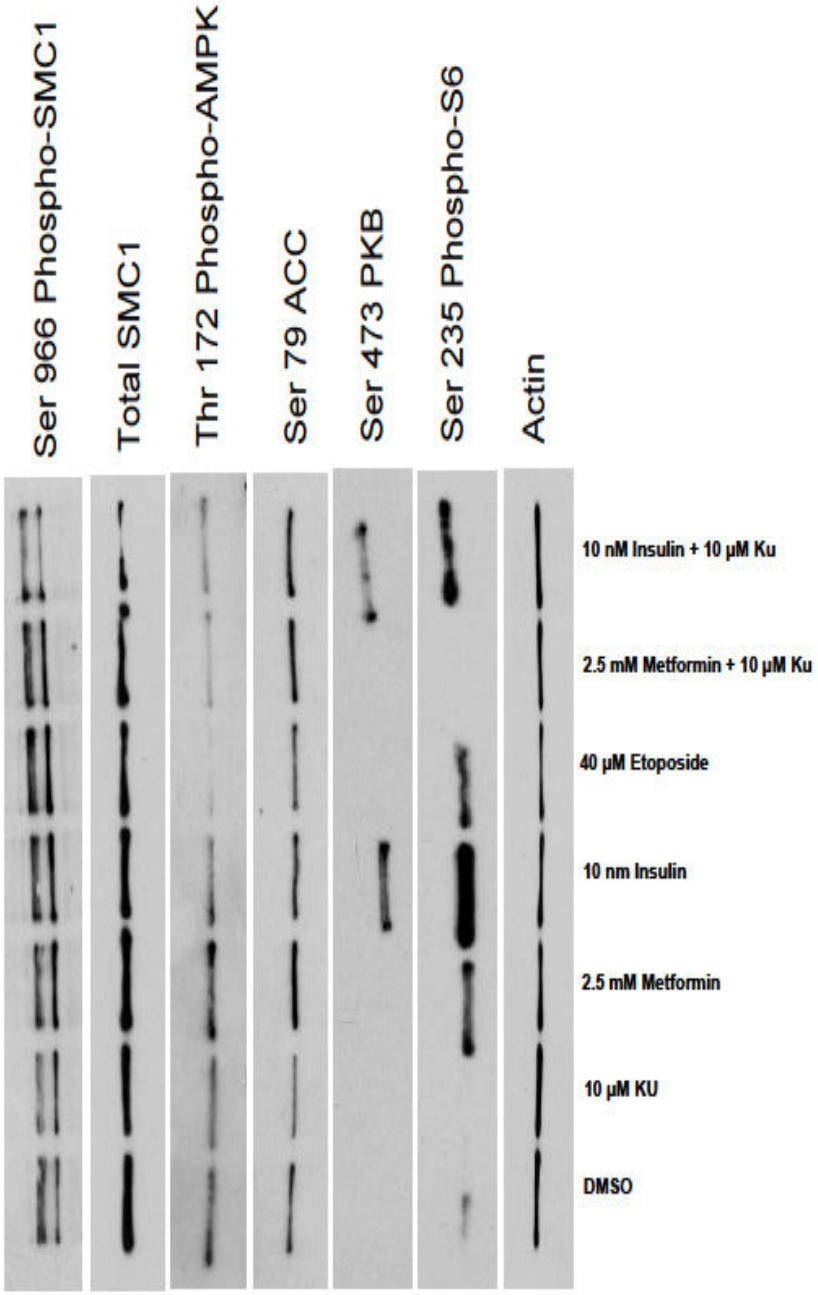


Figure 6.1 Effect of ATM inhibition by Ku55993 on intracellular signalling in H4IIe cells. Cells were fasted for 3 hours prior to pre treatment for 30 minutes with or without inhibitor as indicated, then 1 hour treatment as shown. Analysis by western blotting with the indicated antibodies. (n=2)

Fig 6.1 Densitometry

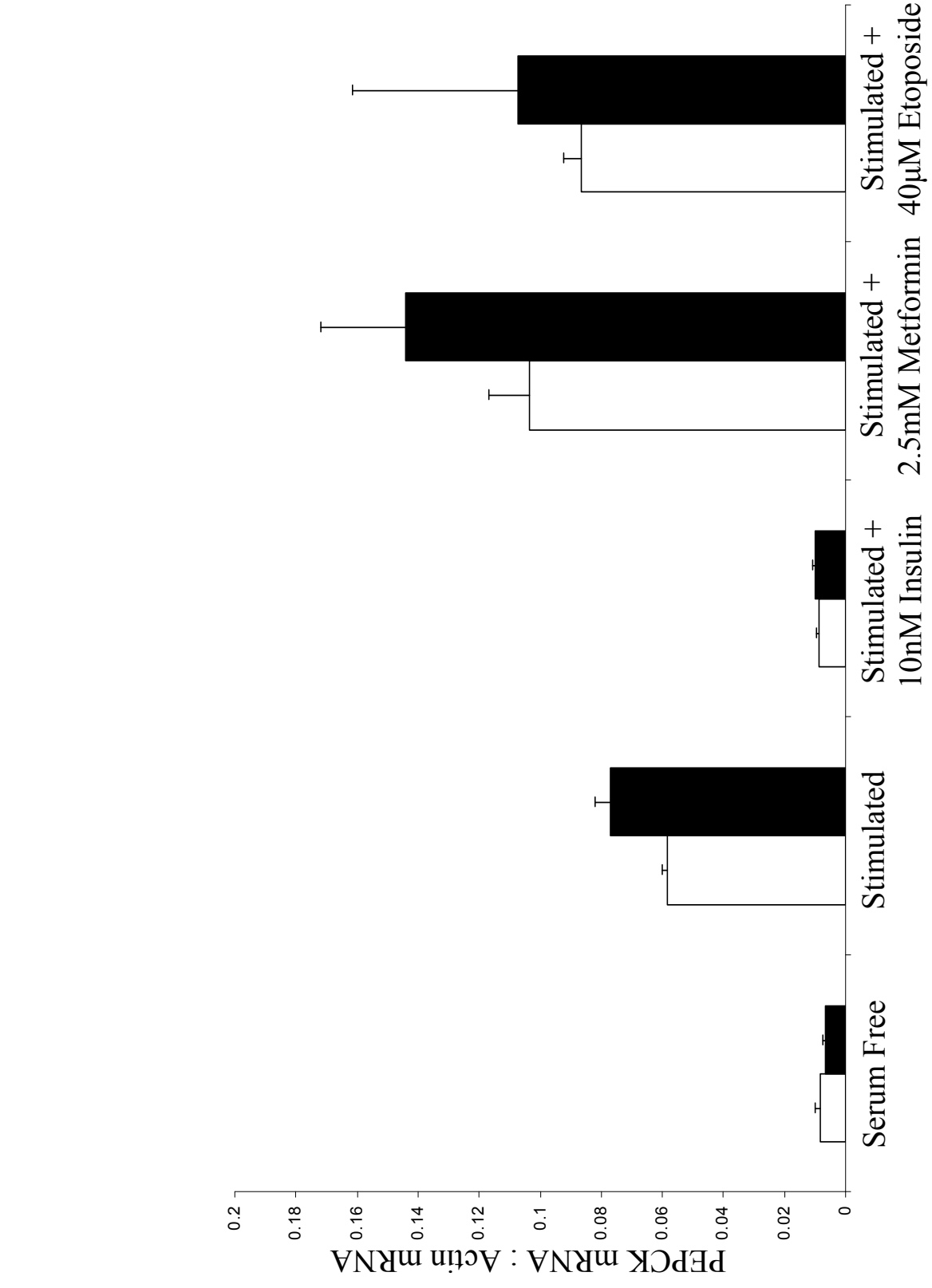


Figure 6.2 There is no effect of Metformin on PEPCK gene expression in H4IIe cells. Cells were fasted for 3 hours prior to treatment for 3 hours (White Bars) or 16 hours (Black Bars) as indicated. Analysis by RTPCR and expressed as ratio of PEPCK to actin mRNA levels. n=2

of PKB was diminished in the presence of Ku55993 (Figure 6.5). Importantly, there was no effect of phenformin, insulin or Ku55993 on the phosphorylation of SMC (Figure 6.5).

Next I assessed the effects of the inhibition of ATM on phenformin regulation of gene expression in H4IIe cells. Cells were treated as in Figure 6.4 with or without the addition of Ku55993. Total cellular RNA was extracted and cDNA synthesised before assessment of mRNA of specific genes by Taqman analysis. Insulin prevented the stimulation of PEPCK expression by dexamethasone and cAMP (Figure 6.6 A), insulin being more effective than phenformin (Figure 6.6 A).The inhibition of ATM, consistent with the reduction of PKB phosphorylation, reduced the effects of insulin on PEPCK expression (Figure 6.6 A). Insulin also prevented the stimulation of expression of IGFBP-1 by dexamethasone and cAMP (Figure 6.6 B). However, phenformin had no effect on IGFBP-1 expression, and in contrast to its effects on PEPCK gene transcription there was no effect of Ku55993 on the insulin regulation of IGFBP-1 (Figure 6.6 B). Similarly, Ku55993 did not affect the action of insulin on G6Pase gene transcription either (Figure 6.6 C). Interestingly, phenformin repressed G6Pase gene transcription in these same cells where no effect on PEPCK was observed, and this effect was blocked by the presence of Ku55993 (Figure 6.6 C). The effects of phenformin and Ku55993 on the PEPCK gene promoter were also examined in reporter cells (Figure 6.6 D). LLRP7 cells were fasted for 3 hours prior to 16 hours stimulation with dexamethasone (500 nM) and cAMP (100 μM 8-CPTcAMP) in the presence or absence of insulin, or phenformin both with and without the presence of Ku55993. The cells were lysed and luciferase activity measured (Chapter 2.20). There was stimulation of luciferase activity with dexamethasone and cAMP, and this stimulation was prevented by both insulin and

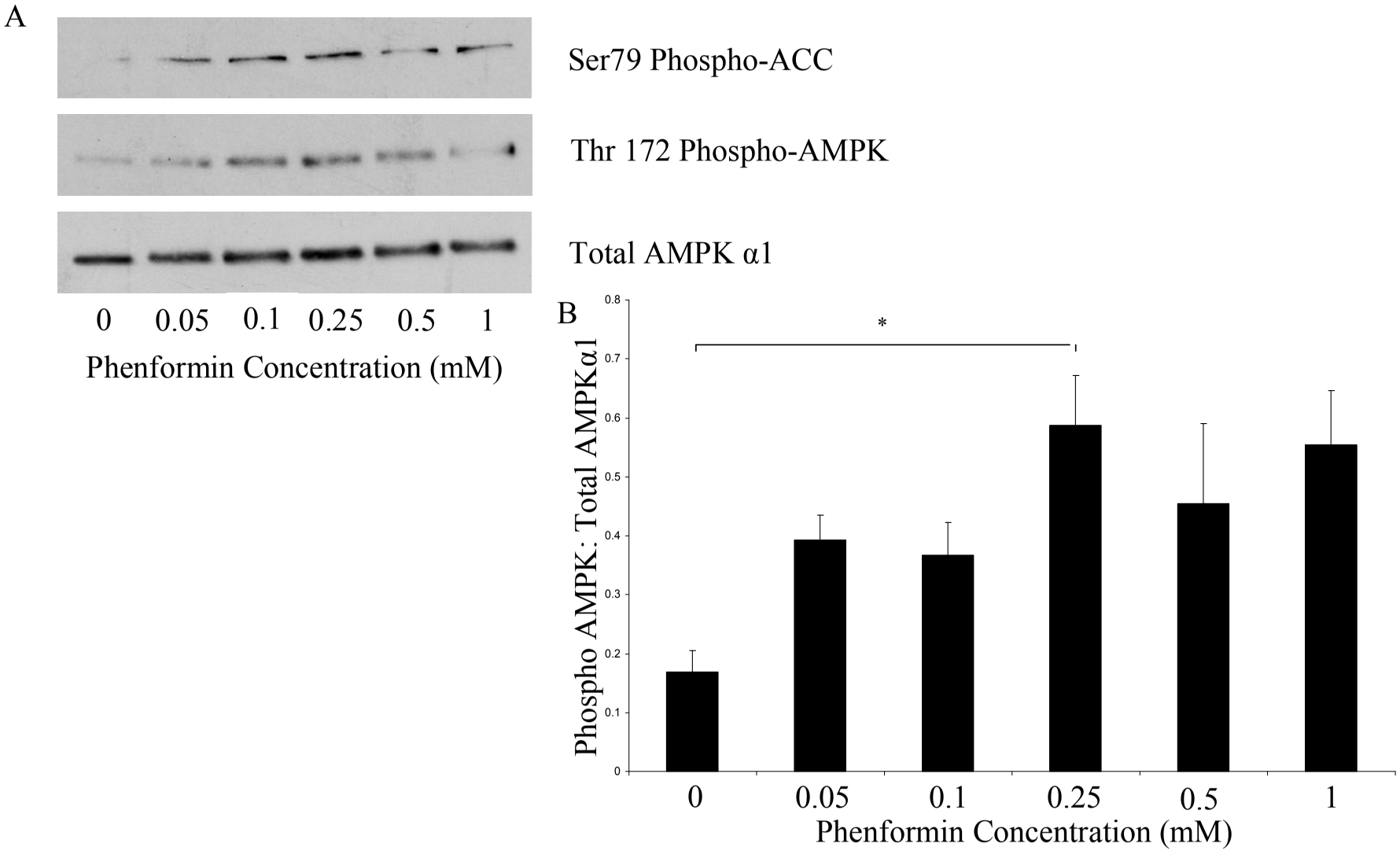


Figure 6.3 Effect of phenformin on AMPK phosphorylation. H4IIe cells were fasted for 3 hours prior to treatment for 1 hour as indicated. (A) Analysis by western blotting with indicated antibodies, representative blot, (B) Densitometry of 3 experiments. *= p=0.001

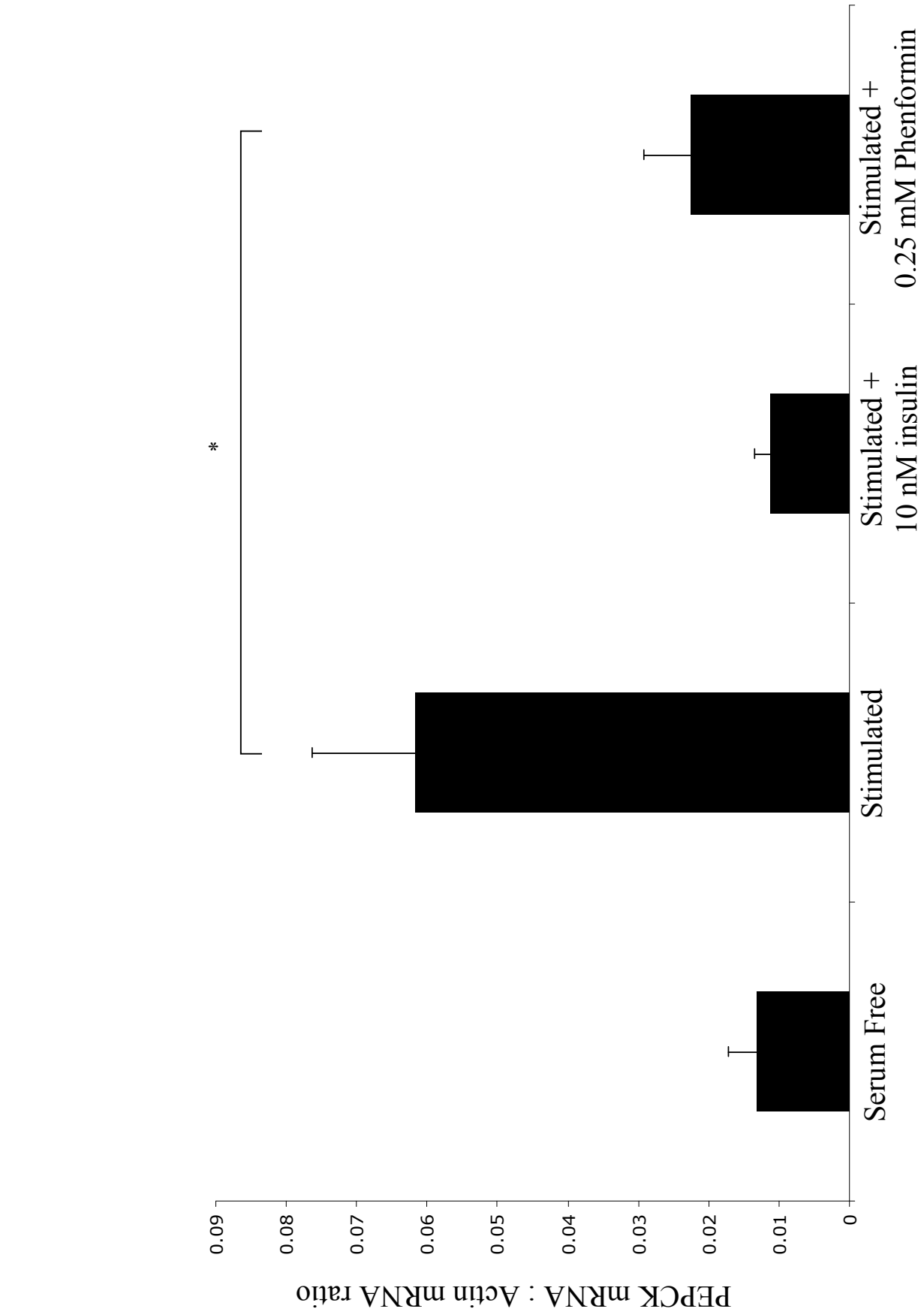


Figure 6.4 Effect of phenformin on PEPCK gene expression. H4IIe cells were fasted for 3 hours before 3 hours treatment as indicated. Analysis of PEPCK levels by Taqman. (n=3-4). p values *=0.02

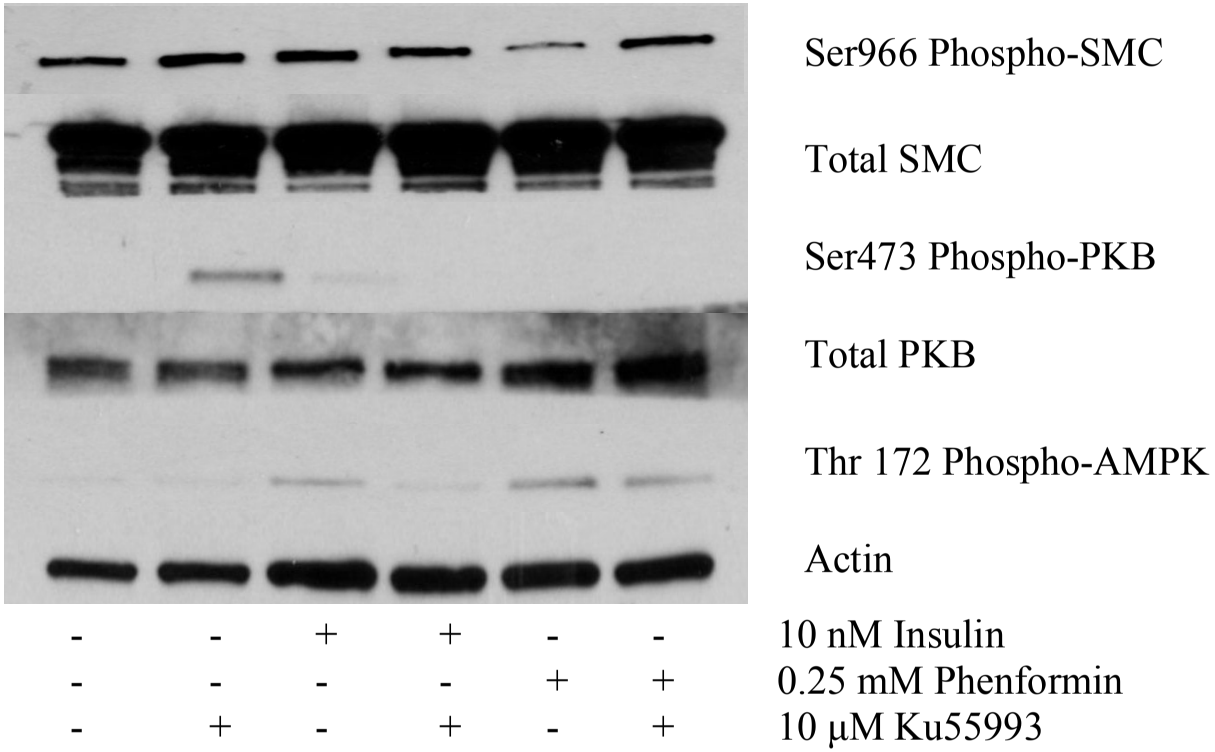
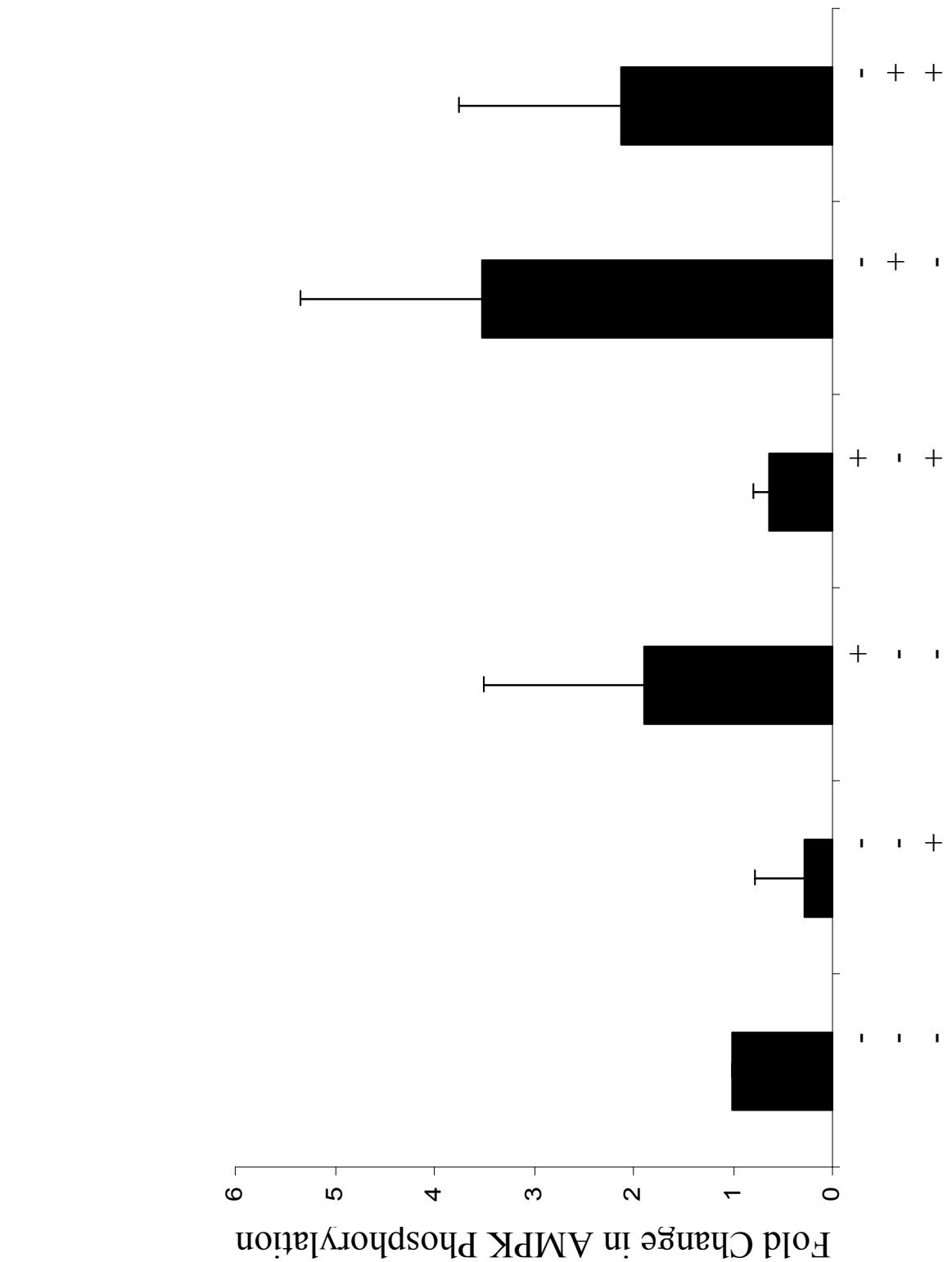


Figure 6.5 Effect of Ku55993 on phenformin induced phosphorylation of AMPK. H4IIe cells were fasted for 3 hours prior to 1 hour treatment as indicated. Analysis performed by western blotting with antibodies as indicated. (A) Representative Blot. (B) Densitometry of AMPK phosphorylation. n=3



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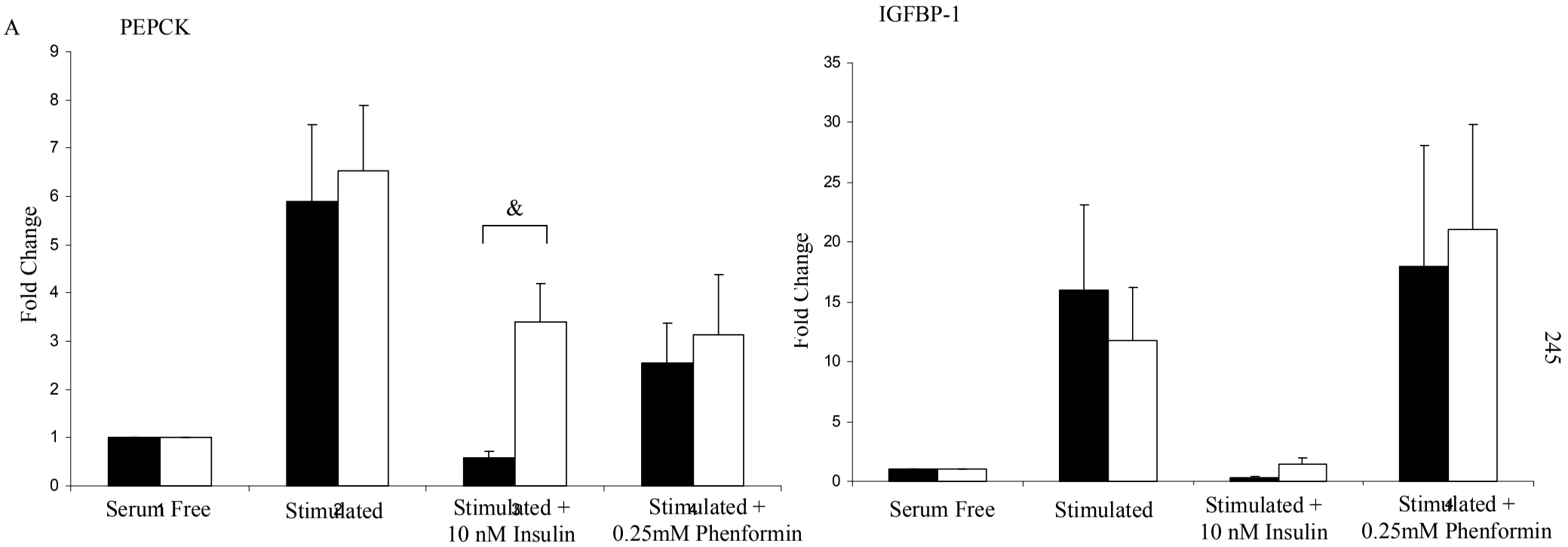

Figure 6.6 Effects of ATM inhibitor Ku55993 on insulin responsive gene expression. Cells were fasted for 3 hours prior to 30 minutes pre-incubation with or without 10 μM Ku55993 followed by either 3 hours treatment as indicated and analysis by TAQMAN of (A) PEPCK, (B) IGFBP-1 (C) G6Pase (n=2 for each gene) or (D) LLRP7 cells treated for 16 hours and analysed by luciferase assay (n=2). Black Bars = DMSO, white bars = 10 μM Ku55993. pvalues & = 0.01, * = 0.05, # = 0.04 .

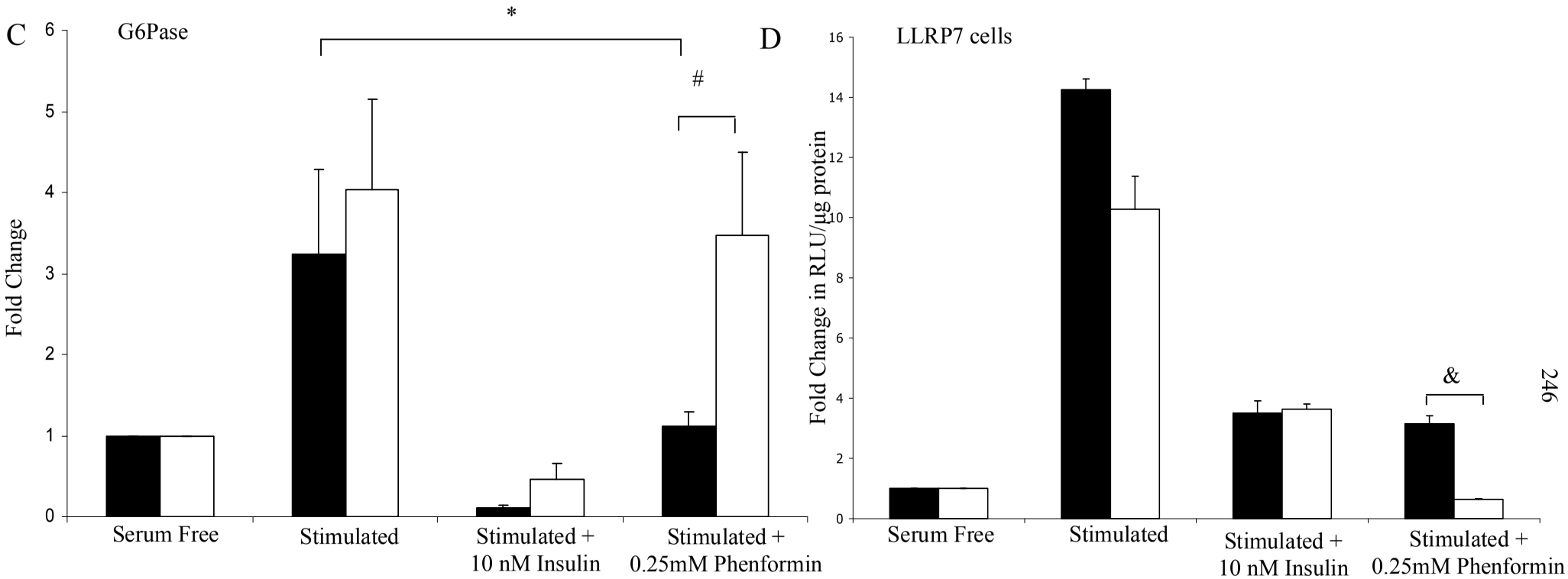

Figure 6.6 continued

phenformin (Figure 6.6 D). In complete contrast to endogenous gene transcription, the effects of insulin on luciferase activity were not reduced by the presence of Ku55993, and the inhibitor enhanced the ability of phenformin to prevent the stimulation of luciferase activity in reporter cells (Figure 6.6 D)

The inhibition of PKB phosphorylation by the ATM inhibitor was somewhat surprising; therefore I investigated the effects of this compound on insulin signalling in more detail (Figure 6.7). H4IIe cells were incubated for 1 hour in the presence or absence of insulin either with or without the presence of Ku55993 (Figure 6.7). The inhibitor prevented the insulin stimulated phosphorylation of PKB at both Thr 308 and Ser473 (Figure 6.7). Ku55993 also reduced the insulin stimulated phosphorylation of S6 (Figure 6.7). However, the inhibitor had no effect on the downstream phosphorylation of p42/44 MAPK (Figure 6.7).

6.2.2. DNA-PK and the regulation of gluconeogenic gene expression

DNA-PK is closely related to ATM in structure, also involved in DNA repair and has been implicated in control of glucose metabolism (Wong et al., 2009). Therefore I decided to check whether hepatic gene transcription was affected by inhibition of DNA-PK (Figure 6.8). H4IIe cells were fasted for 3 hours prior to 3 hours stimulation with dexamethasone (500 nM) and cAMP (100 μM 8-CPTcAMP) in the presence or absence of insulin with or without the addition of 10 μM Nu 7441. Total cellular RNA was extracted cDNA synthesised before assessment of PEPCK, G6PAse, IGFBP-1 and actin levels by Taqman analysis. Dexamethasone and cAMP stimulated the expression of PEPCK mRNA and this was prevented by insulin (Figure 6.8 A). The addition of a DNA-PK inhibitor increased both stimulated and insulin repressed levels of PEPCK mRNA (Figure 6.8 A). However, the inhibitor partly reduced the response to insulin

(80% repression without the DNA-PK inhibitor, and a 47% reduction with it (Figure 6.8 A)). In contrast, inhibition of DNA-PK prevented the stimulation of G6Pase mRNA by dexamethasone and cAMP and therefore it is not possible to assess whether it blocked regulation by insulin (Figure 6.8 B). The effects of inhibition of DNA-PK on IGFBP-1 gene expression were similar to the effects on PEPCK, with the inhibitor increasing both stimulated and insulin repressed levels of IGFBP-1 mRNA (Figure 6.8 C). Once more there was a reduction in the action of insulin with the percentage repression by insulin reducing from 95% to 50% when Nu 7441 was applied (Figure 6.8 C). The effects of Nu7441 on LLRP7 reporter cells (Chapter 4) were similar to the effects on PEPCK mRNA (Figure 6.8 D). LLRP7 cells were fasted for 3 hours prior to 16 hours treatment with dexamethasone (500 nM) and cAMP (100 μM 8-CPTcAMP) in the presence or absence of insulin with or without the addition of 10 μM Nu 7441. Cells were lysed and then assessed for luciferase activity which was corrected for total protein level within each lysate. Dexamethasone and cAMP stimulated the expression of luciferase activity and this was prevented by the presence of insulin (Figure 6.8 D). The inhibitor increased the basal, stimulated and insulin repressed levels of luciferase activity, and almost completely removed any effect of insulin on the suppression of luciferase activity (Figure 6.8 D).

There was approximately a 50% reduction in the protein levels in the sample treated with the DNA-PK inhibitor suggesting that the prolonged exposure to this compound was highly toxic to the cells and making interpretation of data difficult. Therefore, a second DNA-PK inhibitor, DMNB, was investigated to establish whether the cells simply couldn't cope without DNA-PK activity. This compound was less toxic to the cells (as judged by visual inspection of cells) but had little effect on the expression of

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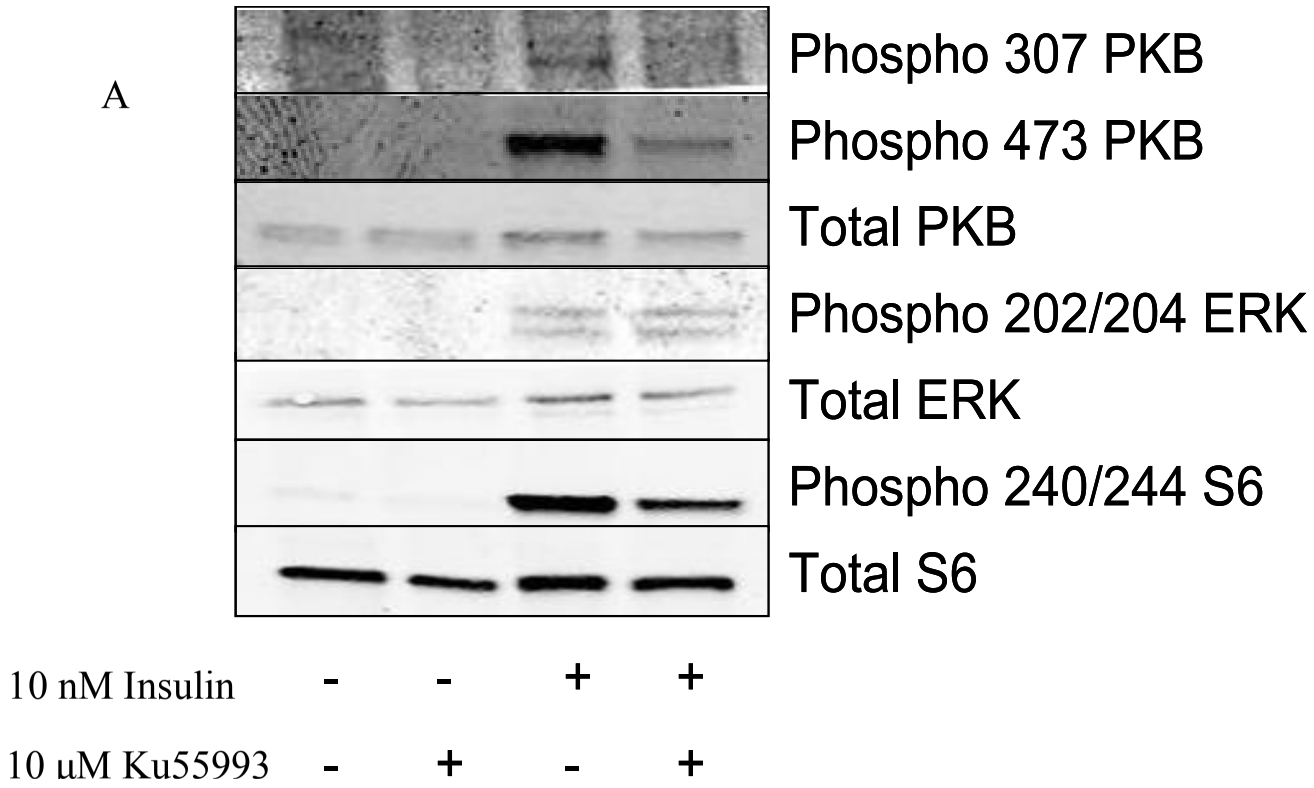
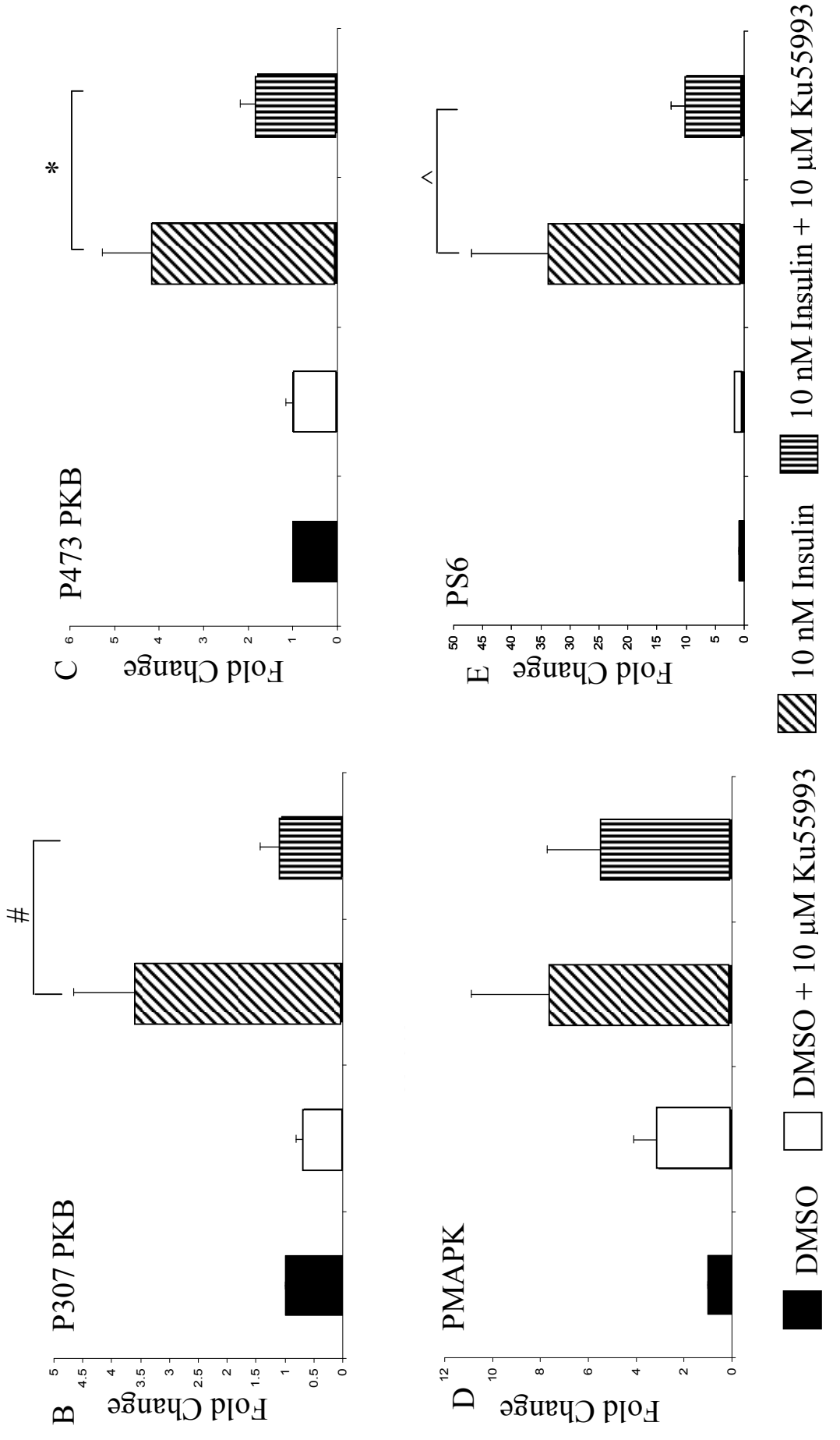


Figure 6.7 Effect of ATM inhibitor Ku 55993 on insulin signalling in H4IIe cells. Cells were fasted for 3 hours prior to 30 minutes pre-treatment with inhibitors followed by 1 hour of treatment in the presence or absence of 10nM insulin. Representative blot of 3 experiments (A) and densitometry of (B), Phospho 307 PKB (C), Phospho 473 PKB, (D) Phospho 202/204 ERK and (E) Phospho 240/244 S6 ribosomal protein. Data shown fold change in relative phosphorylation. P values # = 0.03, * = 0.04, ^ =0.05. n=3



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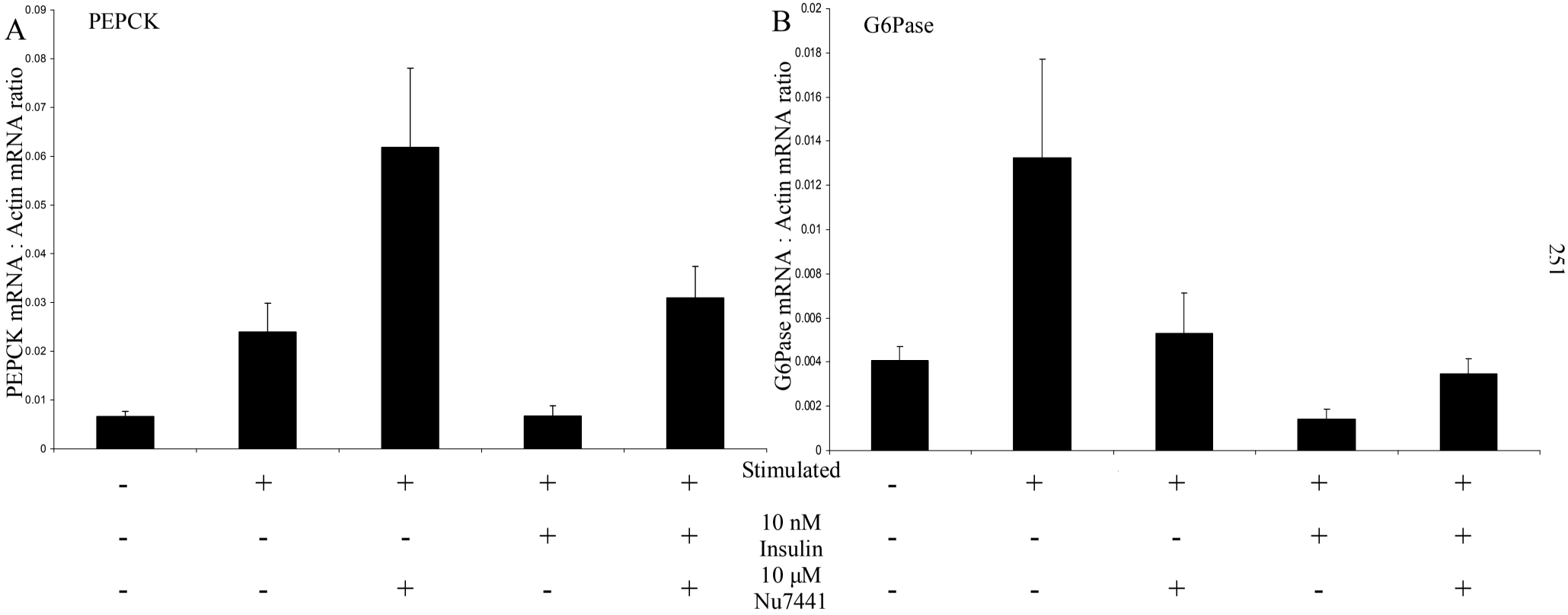
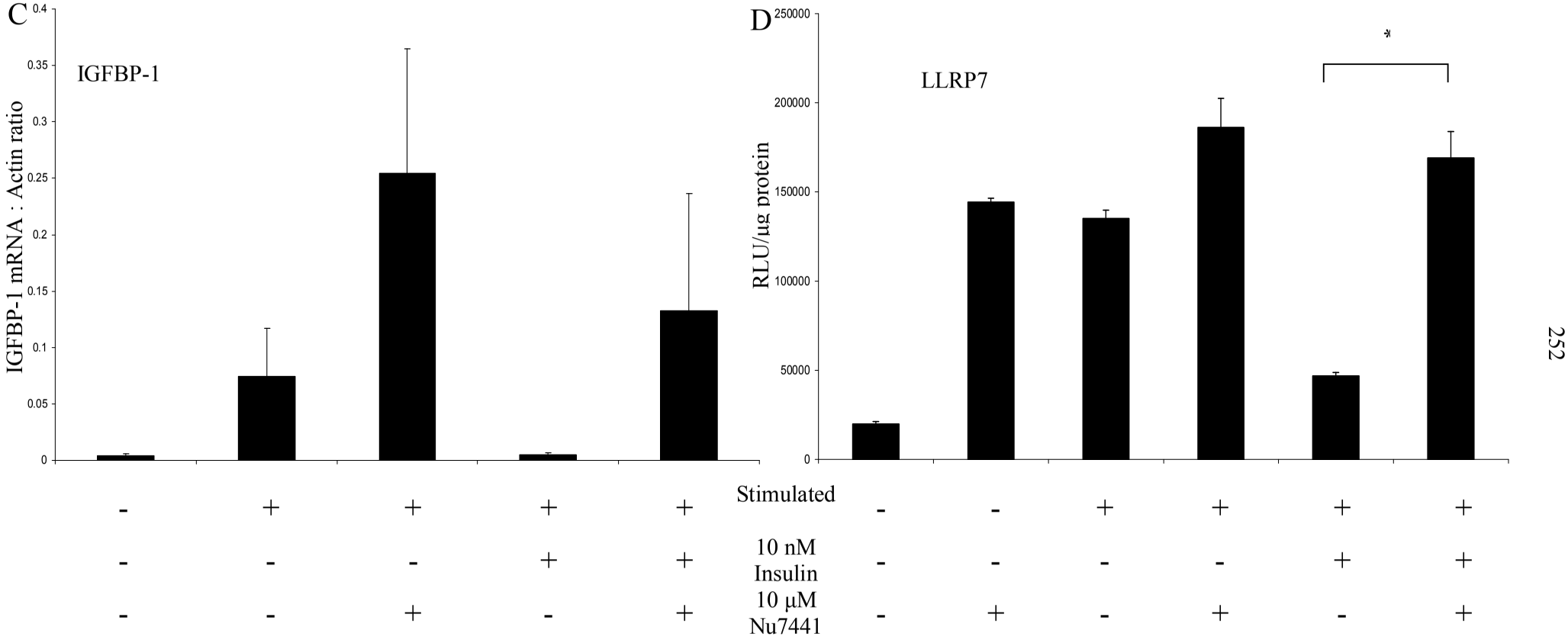


Figure 6.8 Effect of DNA-PK inhibitor Nu7441 on gluconeogenic gene expression in H4IIe cells. Cells were fasted for 3 hours before 30 minutes pre-treatment with or without 10 μM Nu7441 followed by either 3 hours treatment as indicated, analysis by RTPCR of (A) PEPCK, (B) G6Pase, (C) IGFBP-1 or (D) Treatment of LLRP7 cells for 16 hours and analysis by luciferase assay. *, $p<0.0001$. n=4-6



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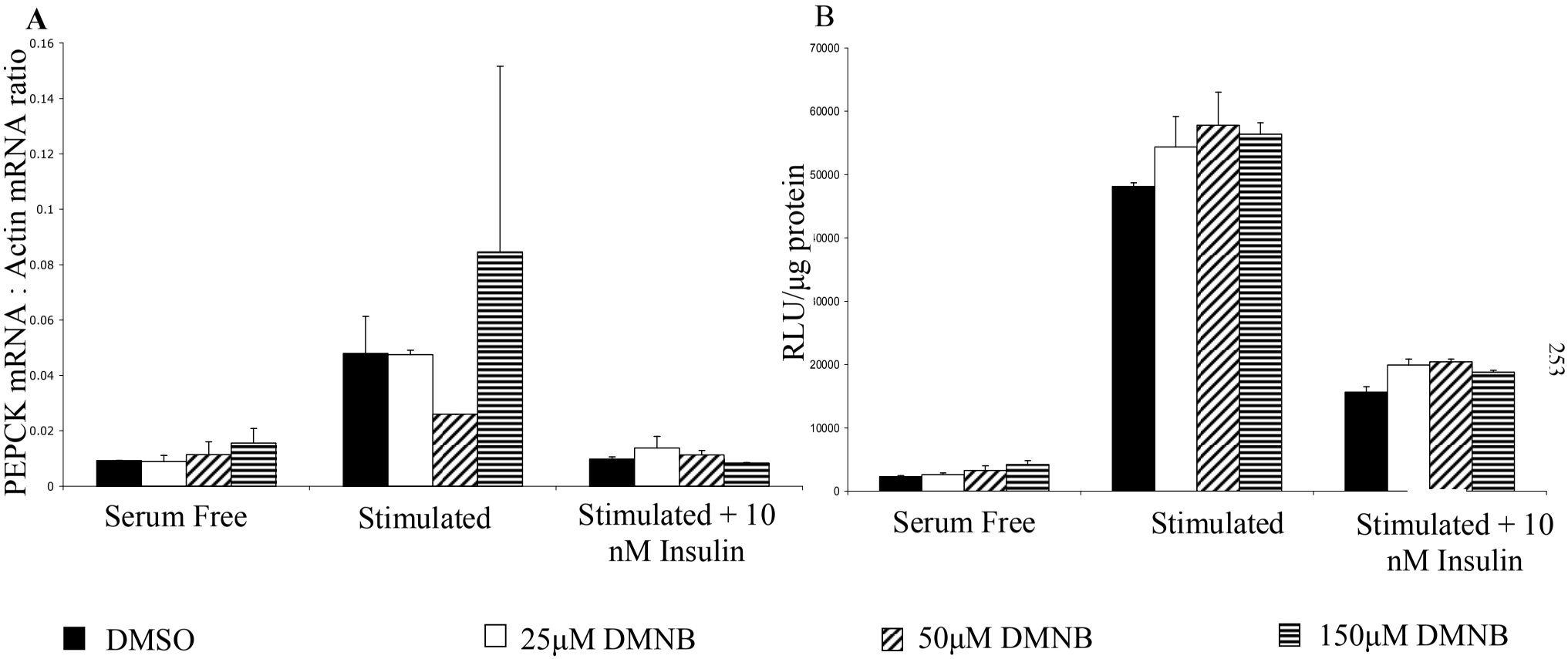

Figure 6.9 DMNB has no effect on PEPCK expression. (A) RTPCR of PEPCK in H4IIe cells. Cells were fasted for 3 hours prior to 30 minutes pre-treatment with the indicated concentration of DMNB then 3 hours treatment as indicated. (B) LLRP7 cells were fasted for 3 hours prior to 30 minutes pre-treatment with the indicated concentration of DMNB followed by 16 hours treatment as indicated. (n=2)

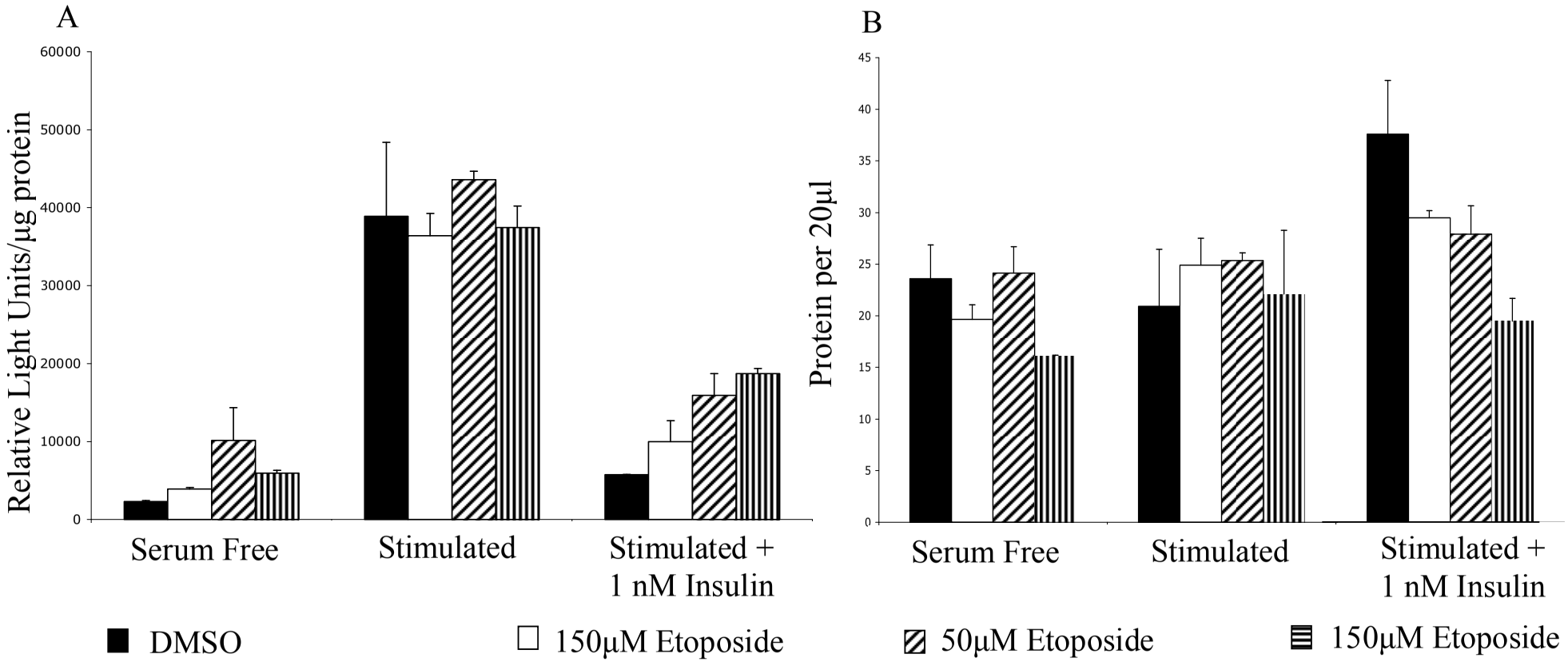

Figure 6.10 Effect of Etoposide on Luciferase activity in LLRP7 cells. (A) Data corrected for protein concentration, (B) effect of etoposide on protein concentration, (C) Raw data not corrected for protein levels. (n=2)

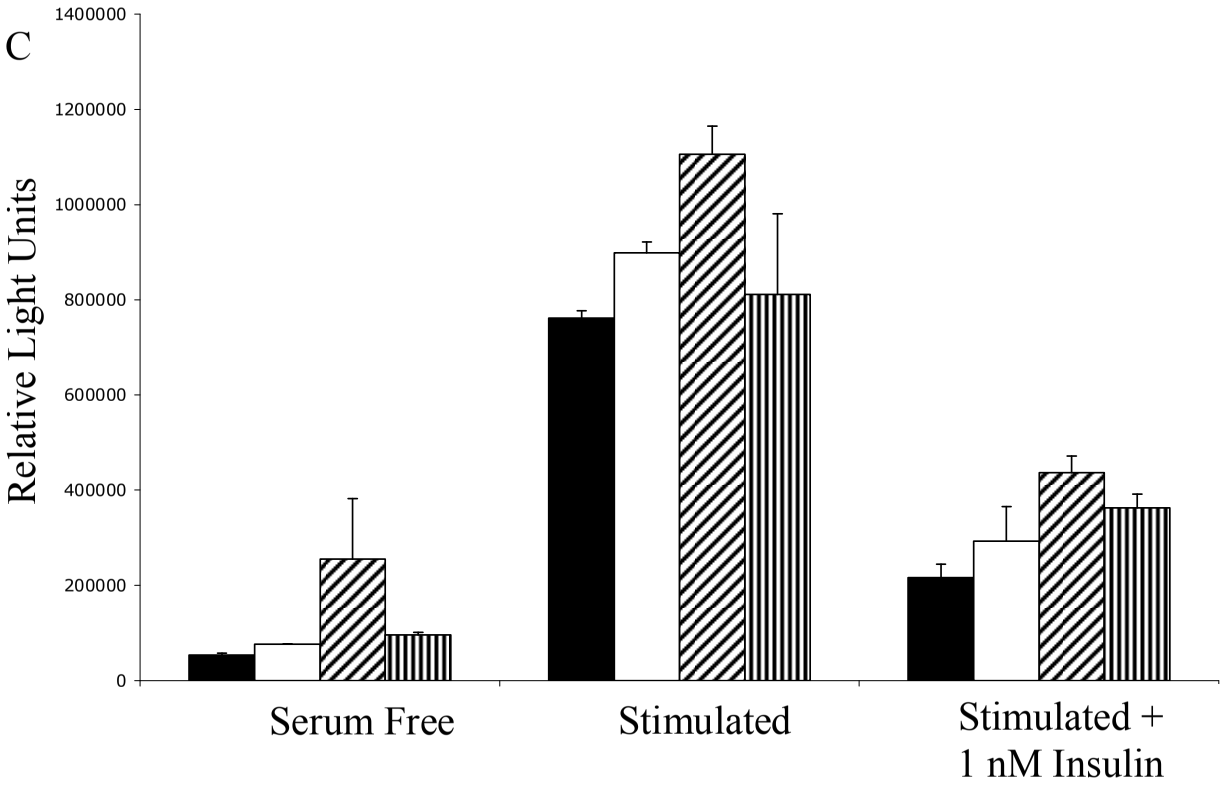

Figure 6.10 continued

PEPCK mRNA at concentrations ranging from 25 to 150 μM (Figure 6.9). In LLRP7 cells, there was no alteration in hormonal regulation of the luciferase activity across the same range of DMNB concentrations (Figure 6.9), and no significant effect on thelevels of protein in the cells. The ATM inducer, etoposide appeared to reduce insulin regulation of the PEPCK promoter in the LLRP cells (Fig 6.10 A) however this agent is known to block protein synthesis. Indeed there was a dose dependent inhibition of cellular protein in response to etoposide treatment of the LLRP cells for 16 hours, most notable in the insulin treated cells (Fig 6.10 B). Therefore if the luciferase activity is not normalised to protein levels, then no effect of etoposide on insulin regulation of the PEPCK promoter is evident (Figure 6.10 C). This makes interpretation of the data difficult, as it is possible etoposide is indirectly antagonising insulin regulation of PEPCK, or is simply reducing insulin stimulation of protein synthesis thereby generating an artefact in the data analysis.

6.3. Discussion

The aim of this chapter was to assess whether the ATM DNA repair kinase (or related kinases) influenced biguanide action on gluconeogenic gene expression.

Metformin is a commonly used insulin sensitizer in the treatment of type 2 diabetes and insulin resistance. Despite the almost ubiquitous use of this drug, the mechanism of action is still unclear. The proposed mechanism of action of metformin in control of glucose homeostasis includes phosphorylation and activation of AMPK (Zhou et al., 2001). Metformin probably induces AMPK as a result of its repressive action on the respiratory chain, promoting accumulation of AMP in cells with metformin transporters (OCT1). AMP allosterically induces AMPK by enhancing its phosphorylation by LKB1 or CaMKK and reducing its dephosphorylation. However, genome wide association studies have demonstrated an association between ATM and the efficacy of metformin in the control blood glucose (Zhou et al., 2011).

Polymorphisms in the ATM sequence could potentially influence metformin regulation of glucose control in multiple ways. Firstly, ATM could lie upstream of AMPK and directly regulate the activity of LKB1, CaMKK or AMPK itself. Alternatively, ATM could influence metformin uptake, its action on the respiratory chain, or the accumulation of AMP. Of course metformin regulation of glucose metabolism could be influenced by ATM activity totally independently of AMPK such as by affecting transcription factors. Finally, it remains possible that the GWAS has identified a polymorphism associated with a gene other than ATM despite the close chromosomal localisation of the polymorphisms to the ATM gene. In support of ATM lying between metformin and AMPK there is evidence that ionising radiation (Sanli et al., 2010) and etoposide (Fu et al., 2008) both induce AMPK activity in an ATM-dependent fashion,

while ATM can phosphorylate LKB1 at Thr 366, and in HeLa cells which lack ATM, this phosphorylation is absent (Sapkota et al., 2002).

Therefore, does the inhibition of ATM alter the regulation of gluconeogenic gene expression by biguanides? Previous work had shown that activation of AMPK by AICAR repressed PEPCK and G6Pase gene transcription (Lochhead et al., 2000), hence it seemed reasonable to assume that if ATM influenced AMPK activation by biguanides that we would detect this using the PEPCK and G6Pase gene promoters as readout. However, more recent work has established that although metformin induces AMPK phosphorylation, this is not essential for its effects on gluconeogenesis (Foretz et al., 2010). Glucose production in hepatocytes is reduced by metformin, even in AMPK null cells (Foretz et al., 2010). Despite this, if ATM lies upstream of AMPK then it should still be possible to observe reduced regulation of AMPK by biguanides, and monitoring gene transcription would add to our knowledge on whether ATM influenced an AMPK-independent effect of biguanides on gluconeogenesis.

I have shown that phenformin treatment of H4IIe cells represses both PEPCK and G6Pase gene transcription, but it does not regulate IGFBP-1 gene transcription. Furthermore, inhibition of ATM prevented the repression of G6Pase but not PEPCK gene transcription by phenformin. Surprisingly, the insulin repression of PEPCK (but not G6Pase and IGFBP-1) was reduced in the presence of the ATM inhibitor. Inhibition of ATM antagonizes insulin induction of PKB phosphorylation in H4IIe cells, suggesting that ATM activation may enhance insulin signaling and this would provide an explanation for the antagonism of insulin repression of PEPCK by ATM inhibition, as the regulation of PEPCK gene transcription is affected by PKB inhibition (Logie et al., 2007). Indeed, ATM has been implicated in the phosphorylation of PKB at Ser473

(but my work is the first demonstration of an effect on Thr 308 phosphorylation), as has DNA-PK (Viniegra et al., 2005, Bozulic et al., 2008). In addition, ATM is required for full activation of PKB and the translocation of GLUT4 by insulin in muscle cells (Halaby et al., 2008). Therefore, ATM may be involved in a number of pathways involved in glucose homeostasis, affecting both the PEPCK response to insulin and the G6Pase response to biguanides. It is not clear why there are differential effects on PEPCK and G6Pase gene transcription, however previous work has found variation in the response of these gene promoters to signalling inhibitors (Lipina et al., 2005). The simplest explanation could be that ATM mediates the insulin receptor connection to PKB, and the metformin connection to AMPK, while PKB activation is not required for insulin repression of G6Pase but is required for repression of PEPCK by insulin. However, previous data implicates PKB in the insulin regulation of both genes (Logie et al., 2007).

In addition, increased ATM activity can downregulate mTORC1 signalling (Cam et al., 2010). Downregulation of mTORC1 with rapamycin alters the metabolism of lipids by blocking insulin stimulated SREBP-1c expression, but rapamycin has no acute effect on insulin repression of PEPCK (Li et al., 2010). In contrast, chronic mTORC1 inhibition with rapamycin leads to insulin resistance and glucose intolerance, effects thought to be mediated in part by enhanced hepatic PEPCK and G6Pase expression (Houde et al., 2010), suggesting that long term loss of this pathway (e.g. by enhanced ATM activity) could contribute to insulin resistance. If correct one could hypothesize that metformin insulin sensitising effects could then be mediated through inhibition of ATM and relief of mTORC1 inhibition. However, there is little evidence for metformin inhibition of ATM in cells. In addition many studies suggest that inhibition of mTORC1 reduces the S6 kinase induced negative feedback on the IRS molecules (see Chapter 1.6.2), thus

current models predict enhanced insulin signalling by mTORC1 inhibition. Consistent with this, adiponectin, which also induces AMPK phosphorylation, and reduces mTORC1 improves insulin signalling (Wang et al., 2007), as does overexpression of a dominant negative form of raptor (Koketsu et al., 2008).

One of the major roles of ATM is in the repair of DNA damage, and it is sensible that there is a close relationship between continued cell growth and proliferation and the energy state of the cell, i.e. it would be foolish for cell growth and proliferation to continue in the presence of either gross DNA damage or energy deficit. Indeed patients with type 2 diabetes have an increased rate of most carcinomas, but a reduced level of prostatic cancer (Hemminki et al., 2010). In cancer cells, there is a switch from reliance on oxidative phosphorylation to dramatically enhanced aerobic glycolysis (Warburg, 1956). This may be mediated by an upregulation of HIF-1 which is also seen in cells that lack ATM (Kim and Kaelin, 2004, Ousset et al., 2010), or by loss of p53 or by induction of the glycolysis-promoting enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase isoform 3 (PFKFB3) (Colombo et al., 2010). Interestingly, there is a reduction in the rates of cancer in metformin treated patients (Evans et al., 2005), and it may be that because metformin switches cellular glucose metabolism back to oxidative phosphorylation (Ousset et al., 2010).

Of course there are many other possible mechanisms that could link ATM and glucose metabolism. For example, nibrin (NBS1) is part of the double stranded DNA repair complex, and its phosphorylation by ATM is essential for repair of double stranded DNA breaks. Phosphorylation can only occur if NBS1 has previously been deacetylated by SIRT1 (Yuan et al., 2007), a protein that also has a key role in the control of gluconeogenesis. The CRCT2-CREB-CBP complex activates PGC1α and leads to

increased gluconeogenic gene expression (Yoon et al., 2001, Koo et al., 2005, Dentin et al., 2007). The deacetylation of CRTC2 by SIRT1 targets CRCT2 for ubiquitin mediated degradation, and therefore subsequent suppression of gene expression that is dependent of PGC1α (such as PEPCK and G6Pase) (Liu et al., 2008). Treatment with metformin increases both SIRT1 levels and activity in a mouse model of diabetes and leads to a reduction in PEPCK and G6Pase gene transcription (Caton et al., 2010). The activation of ATM also increases SIRT1 levels (Wang et al., 2006), and thus SIRT1 induction is associated with metformin action, ATM activity and the repression of PEPCK and G6Pase gene expression. In contrast, SIRT1 has also been implicated in the stimulation of gluconeogenesis in response to nutrient signalling. By directly deacetylating PGC1α and allowing its interaction with HNF4α and FOXO1, SIRT1 leads to the induction of gluconeogenic genes, albeit not under the control of glucocorticoids and glucagon (Rodgers et al., 2005). This differential control may explain how nutrient overload, as one sees in obesity, may lead to increased gluconeogenesis. Different subcellular localisation of SIRT1, or priming by another kinase such as ATM, may go some way to explaining how the same protein can have two different effects, but the precise molecular link between SIRT1 and metformin action remains poorly studied.

Inhibition of DNA-PK stimulated PEPCK and IGFBP-1 mRNA in the presence of cAMP and glucocorticoid and led to a modest reduction in response to insulin. In contrast, there was a reduction in glucocorticoid and cAMP stimulation of G6Pase. If DNA-PK regulates PKB in H4IIe cells by phosphorylating the Ser473 site (Park et al., 2009) then inhibition of DNA-PK would be expected to block insulin repression of PKB, similar to the effects seen with ATM inhibition. However, the toxic effects on the H4IIe

cells of the inhibitor employed made it difficult to interpret any effects on gene transcription.

6.4. Conclusions

In conclusion, studies with a selective inhibitor of ATM suggest it plays a role in the control of gluconeogenic gene expression, either in the response to insulin (PEPCK) or biguanides (G6Pase). This adds further credence to the GWAS identifying ATM as a 'metformin action gene'.

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Chapter 7. Conclusions and Future Perspectives

The work presented in this thesis has contributed to the understanding of the development of hepatic insulin resistance and the generation of tools that may aid the search for effective therapeutics for the prevention of T2DM. I have characterised an insulin resistant cell model as defined by a reduction in the ability of insulin to repress cAMP and glucocorticoid stimulated gene expression. The model was originally developed using serum from obese rats (Logie et al., 2010) but I have extended the work and made it more clinically relevant by culturing in serum from human diabesity patients. Furthermore, the insulin response of the cell model was relatively accurate at diagnosing sera as case or control; indeed it was more effective than any single biochemical marker, including glucose, at identifying cases and controls (Table 3.3). However, there was not a linear relationship between the generation of insulin resistance *ex vivo* in culture by sera and any single serum or clinical factor, suggesting there is more than one factor in the sera contributing to generation of hepatic insulin resistance in culture. That said, I found that chronic exposure of cells to slightly elevated insulin concentration can affect the insulin sensitivity of the cells to a similar degree as complete diabesity sera. This points towards insulin as a significant promoting factor in the development of insulin resistance. It is of note that the development of insulin resistance in these experiments is not the complete loss of insulin action as one may see in other models of insulin resistance, but a shift in the potency of insulin action as observed in the pathophysiological state.

Unfortunately, I was not able to identify the intracellular changes associated with the reduced insulin action in the cells. In particular there were no defects in the classical IRS-PI 3-K-PKB insulin signalling pathway, and since almost complete inhibition of PKB is required to alter the response of PEPCK gene transcription to insulin this argues against the diabesity sera effects being due to alterations in PKB signalling. Presumably

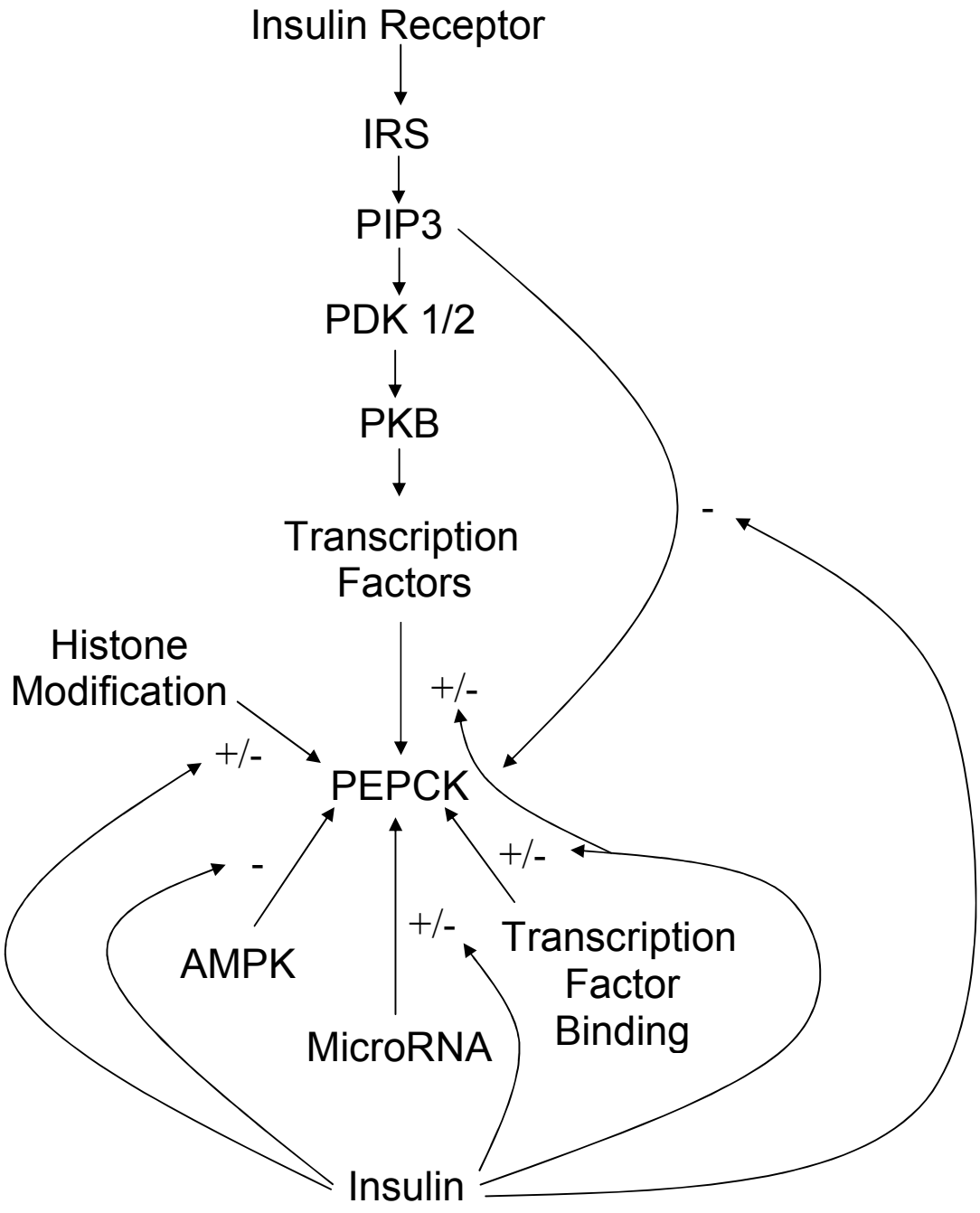

Figure 7.1 Diagram showing potential sites where enhanced insulin may be reducing the ability of insulin to acutely regulate PEPCK mRNA. Decreased effect on signalling/regulation of PEPCK=-, increased or decreased effect on signalling/regulation of PEPCK=+/-

there are other pathways involved in the insulin repression of gluconeogenic genes. Future work could investigate other potential pathways to the PEPCK gene promoter, such as oxidative stress mediators, AMPK pathway, a pathway downstream of PI-3K but not involving PKB, microRNA, and histone modifiers, all within cells cultured in the presence of these higher levels of insulin (or diabesity sera) (Figure 7.1). The use of chromatin immunoprecipitation to establish changes in the binding of specific transcription factors to the PEPCK promoter would establish the endpoint changes associated with reduced insulin response. Unfortunately the factors that mediate insulin repression of this promoter have not been completely elucidated but PGC1α, FOXO, HNF3B, TORC and CBP are worthy of investigation.

Due to time constraints I was unable to assess the effects of other constituents of diabesity serum on the development of insulin resistance in hepatoma cells. Future investigation would include culturing in serum containing leptin or glucose, or depleted of adiponectin, to the concentrations seen in the serum of our volunteers. If a similar change in insulin sensitivity was also seen by chronic exposure to leptin or glucose, it would indicate a redundant role of each of these components in the development of resistance and nullify any intervention designed to target only one component (i.e. hypoglycaemic agent that does not reduce leptin or insulin). This information would also permit the "manufacture" of a resistance causing serum by mixing all of these components, allowing future investigation of signalling changes and novel insulin sensitisers. However if only one component is responsible for the observed effect of the diabesity sera then that becomes the key therapeutic target.

A weakness of the current work is the lack of data on sera from obese (insulin resistant), normoglycaemic individuals and from lean diabetic individuals. These populations

would permit clearer assessment of whether the resistance developing in response to culture in diabesity sera was more related to obesity or diabetes (both of which have associations with hyperinsulinaemia, so both could still generate resistance in our model). This work is key to evaluate potential target patient populations for therapeutics developed using this screen. In theory separate cell models generated using sera from each group could identify different lead compounds for improvement of insulin sensitivity.

Unfortunately, culturing LLRP7 cells in the serum of insulin resistant humans did not alter insulin sensitivity of the transgene, despite it containing all of the gene promoter elements required for insulin repression of the PEPCK gene promoter. The reasons for this are not known, but it may be related to the longer experimental time required for luciferase production (compared to mRNA generation), the subsequent stability of luciferase (as we are measuring the negative effect of insulin on transcription if the protein is stable there would be a lag before detection of changes in protein) and, we had the complication of an off-target stimulation of luciferase production by insulin mediated possibly by a vector element. In addition, we did not assess the number or position of copies of the transgene inserted into the cellular genome of each reporter cell, all of which could affect the basal and stimulated expression of luciferase. There are ways to attempt to address each of these possible problems, for example, introduction of a destruction domain within the luciferase to increase its turnover, the generation of isogenic clones at a targeted location of the genome, (potentially even a knockin of a bicistronic PEPCK-luciferase gene in the position of the endogenous PEPCK gene). Our colleague, Dr Sakamoto is investigating infection of mice with an adenoviral construct including the PEPCK gene promoter luciferase construct, and this will help establish

reporter constructs that are less sensitive to insulin in obese mice (as these vectors do not integrate into host genome).

In conclusion, the work presented in this thesis shows that the serum from human diabetic subjects is able to induce insulin resistance in rat hepatoma cells and that chronic, albeit mild, hyperinsulinaemia may be sufficient to recapitulate this property of the serum. The importance of this finding is that it provides additional evidence that a defect in insulin secretion (producing even mild hyperinsulinaemia) may be a major cause of insulin resistance, placing β-cell problems earlier in the etiology of type 2 diabetes than many believe. Indeed one could argue that if mild hyperinsulinaemia affects insulin sensitivity of hypothalamic neurons it could lead to or enhance hyperphagia and adiposity, hence beta cell defects could arise prior to obesity as well as clinical insulin resistance. These questions on the time line of development of endocrine and metabolic defects are crucial to the development of appropriate therapeutics to combat the underlying cause of type 2 diabetes rather than just the symptoms. One hopes that clinically relevant cell and animal models can be developed to accelerate the discovery of such agents before the diabesity epidemic cripples our health service.

Appendix 1

Inclusion Criteria

Patients must fulfil all of the following criteria:

Obese Diabetic group

- Diabetes (diet treated)
- Hba1c ≤ 8%
- Male
- White European
- Age >35 and <60
- BMI ≥ 30
- Non−smoker
- No treatment with fibrate or anti-diabetic medication

Control group

- Male
- White European
- Age = >35 and <60
- BMI < 27
- Non−smoker
- No treatment with fibrate medication

Exclusion Criteria

Patients are to be excluded from the study if they meet any of the following criteria:

Obese Diabetic group

- HbA1c >8%
- Female
- Age ≥ 60
- BMI <30
- Smoker
- Concurrent treatment with fibrates or anti-diabetic
- Family history of type 1 diabetes (defined as relative developing diabetes and commencing insulin treatment within 6 months)
- Non White−European

Controls.

- Diabetes
- Female
- Age ≥ 60
- BMI > 30
- Smoker
- Concurrent treatment with fibrates
- Family history of diabetes in first degree relative
- Non White−European

Appendix 2

Rat PEPCK sequence

GTACGAGCGCTGAACATCACACAAGGTGGGCTGGGAGCTCTTTGGGGAGTC

CTAAGAGGGCAGCTGGCAATGGACACCTAGCAGTCCCTTTGAGACTTATTTC

AGATGGAGCTGTAGAAAGATGCCATGGCTCACAGTGCCTCCCTGGGAAGGG

GGCAGAGGGCTGCCCAGTGAGGCCTCTTGCGAGCAGGAAATCACCAGAGAC

AAGGAAAGACCAGACCCCAGGATGACCTCAGTTAGGCCTTGCCCGACTGTC

CTCAGAGTCCCATTCTCTGTGTCCTGGTTCTTTTAGAAGATCATGGACCTCC

AGGTCATTTCGTAACCGGAATCTGCCTTGCGGGGGGTTTTGACAAGCTATGG

TATAGTGTATGTGGGGGTACTGACGAATTGGAAGATCATGGAGACCCCTTC

TCCTCCTCCATCATTGGTCTGCCACATCCCTCCCAGGAGACTCACAGCAGAG

AGACCTTGGATGTATGTAGGGTGCTTTAAAACTCCAGCTGAGTTACAGTCTC

TCCTTTCTGTTTTCACCTTAACCTTCCAGGGATGCAAACCCACGACAGGTTT

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AGGCTGGTTTAGCCCAGGCCTGATGTGGAGGCACTGAGCTGGACGTTCTAG

CGGGGTTGACACCCAACAGTTTACATAGGGGGAGGCCACCCCTCCTGAGCA

GTCTCGGTGACTTGAAGAGGAAGCCGCTTCTTCTGTACCAACACAGAAGCT

CCAGCGAACCCCCAGAATGCTGGCAGTGTGGGTGCTATGTAAAAGTATTTA

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CGTGAAGGTCTGTGTCTTGCAGAGAAGGTTCTTCACAACTGGGATAAAGGT

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CACCCCAGCCAACTCGGCTGTTGCAGACTTTGTCTAGAAGTTTCACGTCTCA

GAGCTGAATTCCCTTCTCATGACCTTTGGCCGTGGGAGTGACACCTCACAGC

TGTGGTGTTTTGACAACCAGCAGCCACTGGCACACAAAATGTGCAGCCAGC

AGCATATGAAGTCCAAGAGGCGTCCCGGCCAGCCCTGTCCTTGACCCCCAC

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AAGTTTAGTCAATCAAACGTTGTGTAAGGACTCAACTATGGCTGACACGGG

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GGGCAGGGCTGTCCTCCCTTCTGTATACTATTTAAAGCGAGGAGGGCTAGCT

ACCAAGCACGGTTGGCCTTCCCTCTGGGAACACACCCTTGGCCAACAGGGG

AAATCCGGCGAGACGCTCTGAGATCTCTGATCCAGACCTTCCAAAAGGAG

Human PEPCK sequence

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CACACTGCAAAGTCCACTCGGCGGGTTAACTGGGGGTCCTCAGGGATCCCA

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ACTGACCCTGGCCGTGATCCAGAGACCTGCCCCCTGACGTCAGTGGCGAGC

CTCCCTGGGTGCAGCTGAGGGGCAGGGCTATTCTTTTCCACAGTATTTAAAG

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CTGGCGGGAAGAGCCCGGAAAGAAACCTGTGGATCTCCCTTCGAGAT

Human G6Pase Sequence

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CCTCACCTGTTTTCCCACGGATAAAAACGTGCTGGAGGAAGCAGAAAGGGG

CTGGCAGGTGGAAAGATGAGGACCAGCTCATCGTCTCATGACTATGAGGTT

GCTCTGATCCAGAGGGTCCCCCTGCCTGGTGGCCCACCGCCAGGAAGACTC

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Human IGFBP1 sequence

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CTAACCCCATCTCGCCTTTCCTCACCTGGGGCATTGTTTTCTGCGTTTGAGAA

CTGCTGGGTGGGCTGGTGTTACCCAGGATTTTTAAATTTTTGCAGGAGACGC

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CTCCTAGCGTGCGGCGCTGCCAATCATTAACCTCCTGGTGCAAGTGGCGCGG

CCTGTGCCCTTTATAAGGTGCGCGCTGTGTCCAGCGAGCATCGGCCACCGCC

ATCCCATCCAGCGAGCATCTGCCGCCGCGCCGCCGCCACCCTCCCAGAGAG

CACTGGCCACCGCTCCACCATCACTTGCCCAGAGTTT

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